PROJECT PLAN FOR THE BIOLOGICAL EFFICACY TESTING OF THE XXXXXXXX BALLAST WATER TREATMENT SYSTEM FROM XXXXXXXX GmbH AS PART OF THE TYPE APPROVAL PROCESS UNDER RESOLUTION MEPC.174(58)

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L. Peperzak Ph.D.

NIOZ Royal Netherlands Institute for Sea Research
P.O. Box 59
NL-1790 AB Den Burg, The Netherlands

Abstract

This project plan for the testing of the Xxxxxxxx Ballast Water Treatment System consists of the following sections:

1. Quality Management Plan (QMP)
2. Quality Assurance Project Plan (QAPP)
3. Standard Operating Procedures (SOPs)

The Quality Management Program (QMP) addresses the quality control management structure and policies of the test facility.

The Quality Assurance Project Plan (QAPP) in section 2 is the project-specific technical document reflecting the specifics of the test facility, the BWTS tested, and other conditions affecting the actual design and implementation of the required experiments. The QAPP consists of general information, information on the NIOZ test facility and the Xxxxxxxx BWTS, test water quality, sampling and sample storing, the measurement of variables and QA/QC.

Detailed Standard Operating Procedures (SOPs) for each of the analyses are provided in section 3.

This is the second version of the project plan. The first version contained information for intermediate and high salinity range testing only. This new version includes information on freshwater testing scheduled for autumn 2012 and is split in the three sections outlined above.

Section 1: Quality Management Plan (QMP)

Section 2: Quality Assurance Project Plan (QAPP)

Section 3: Standard Operating Procedures (SOPs)

Variable	Reference (SOP)	Page
Salinity and Temperature	Salinity and Temperature 2012.2	46
pH	pH 2012.1	47
TSS and Particulate Organic Carbon	TSS-POC 2012.2	48
Dissolved Oxygen	Dissolved Oxygen 2012.1	50
Dissolved Organic Carbon	DOC 2012.1	53
Viable organisms ≥50 µm, including diversity	Mesozooplankton 2012.1	57
Phytoplankton (organisms 10-50 µm)	Phytoplankton Canto FCM 2012.1 FCM Canto operation 2012.1 FCM Canto data processing 2012.3	59, 61, 64
Phytoplankton diversity	Koeman & Bijkerk b.v.; quality assessments in www.planktonforum.eu/	Koeman & Bijkerk, outsourced
Phytoplankton vitality (PAM fluorimetry)	Phytoplankton vitality PAM 2012.1	66
Phytoplankton vitality (SYTOX Green)	Phytoplankton vitality SYTOX FCM 2012.1	68
Phytoplankton viability	Plankton viability T5-incubation	71
Microzooplankton (organisms 10-50 µm) including diversity	Microzooplankton 2012.2	69
Microzooplankton viability	Plankton viability T5-incubation	71
Phytoplankton (organisms <10 µm)	Phytoplankton Canto FCM 2012.1 FCM Canto operation 2012.1 FCM Canto data processing 2012.3	59, 61, 64
Heterotrophic bacteria	Bacteria count PicoGreen 2012.1	73
E. coli	NEN-EN-ISO 9308-1	Eurofins C-mark, outsourced
Enterococci	NEN-EN-ISO 7899-2	Eurofins C-mark, outsourced

Section 1: Quality Management Plan (QMP)

1. Introduction

The NIOZ Ballast Water Treatment System (BWTS) test facility is part of the Department of Biological Oceanography of the Royal Netherlands Institute for Sea Research (NIOZ). NIOZ is an institute liaised to the Netherlands Organization for Scientific Research (NWO). The mission of NIOZ is to gain and communicate scientific knowledge on coastal seas and oceans for a better understanding and sustainable use of our planet, to manage the national facilities for sea research and to support research and education in the Netherlands and in Europe.

NIOZ is an independent academic research institute participating in numerous international research projects in coastal seas and the oceans. In addition, time series of physical, chemical and biological data from the Wadden Sea area are maintained in order to study long-term changes in the ecology of this UNESCO World heritage site. In this context the research on the efficacy of Ballast Water Treatment Systems (BWTSs) and related environmental questions fits within the NIOZ work field. NIOZ continues to build on its many years of experience: since 2007 NIOZ has tested nine BWTSs (Table 1).

Table 1. Ballast Water Treatment Systems tested at NIOZ.

year	Company	System
2007	Hamann	SEDNA
2008	EcoChlor	EcoChlor
2008	Hyde Marine	Hyde Guardian
2009	Mahle	Ocean Protection System
2009	Severn Trent De Nora	BallPure STDN
2010	Aquaworx	AquaTricomb
2010	Erma-First ESK SA	ErmaFirst
2011	Hamworthy/Wärtsilä	Aquarius-EC
2012	Hamworthy/Wärtsilä	Aquarius-UV

NIOZ works constantly on improving its methods and technologies for the accurate and precise enumeration of aquatic organisms and the measurement of their vitality and viability. These methods are compared with other organisations and test facilities in workshops and within the global network of BWTS test sites, GloBal TestNet, of which NIOZ is a member. On a European level NIOZ is the lead beneficiary of the North Sea Ballast Water Opportunity project. It is policy of the institute to communicate scientific results as much as possible through international peer reviewed publications.

The NIOZ BWTS test facility not only performs land-based G8 or G9 tests. It is also equipped for laboratory tests to investigate the principles and efficiency of new technologies as well as for bench-scale tests to examine prototypes and components of BWTSs. In addition, the test facility is able to analyse and evaluate samples from other test facilities, from ship-board tests and other ballast water related activities. This evaluation may range from compliance checking to IMO standards till advanced statistical analyses. In co-operation with IMARES (Den Helder, The Netherlands) and Go-Consult NIOZ also offers a one-stop shop for complete G8 or G9 type approval, including toxicity testing, ship-board tests and dossier formation. In all activities the NIOZ test facility strives to achieve highly accurate and precise results.

This Quality Management Plan (QMP) describes the organisation of the NIOZ quality system. This consists of the quality system management and organisation, the quality system components, personnel qualifications and training, procurement of items and activities, documents and records, computer hardware and software, planning, implementation of work processes, assessment and response and, finally, quality improvement.

2. Quality system management and organisation

The quality system of the NIOZ is devoted to the reliable testing of BWT systems. This means that accurate and precise measurements of the test ballast water and the treated water, including the experimental control water, need to be assured. Accessibility of

experimental and measurement protocols, quality control, data and data analysis should lead to a high fidelity in the conclusions reached during G8/9 tests of BWTS performance and other relevant research activities. NIOZ is an IMO recognised test institute and is certified by Lloyd's Register.

The project leader of ballast water research is Dr. Louis Peperzak. He is responsible for the proper functioning of the test facility and for carrying out BWTS tests, laboratory analyses, production of Standard Operating Procedures (SOPs), new methods in ballast water research (treatment, enumeration, vitality/viability measurements, compliance monitoring) and the support of all staff and students that are involved in this research. In cooperation with members of the ballast water team he analyses the BWTSs test results and writes the G8/G9 reports as first author.

Co-project leader is Dr. Jan Boon. He manages the overall Ballast Water Project at NIOZ, especially in relation to the North Sea Ballast Water Opportunity (NSBWO) project of the European Union of which the NIOZ is the lead beneficiary. He is also responsible for the external relations of the Ballast Water Project. Because of his experience in chemical quality assurance programs, Dr. Boon also acts as the quality manager. He is responsible for the QMP and will evaluate all QA/QC activities of BWTS tests. He is co-author of the G8/G9 reports.

Both project leaders are assisted by Mrs. Dörte Poszig M.Sc. M.A. (general management), Mr. Marcel van der Linden (financial management) and Mrs. Marieke Holthuijsen-Vloemans (external communication).

Sampling, sample analyses and first data analyses are carried out by Mrs. Josje Snoek, Mrs. Eveline Garritsen, Mrs. Eva Immler, Mr. Dennis Mosk and Mr. Alex Blin. Mrs. Eva Immler is responsible for the correct operation of the first NIOZ test installation on the Pelagia quay and for correct sampling, sample handling and storage there. Mr. Dennis Mosk is responsible for the correct operation of the NIOZ test installation on the Navicula quay and for correct sampling, sample handling and storage there.

The operation and maintenance of both NIOZ test installations is contracted to Mr. A. Smit of Smittech (Den Hoorn, The Netherlands) and Mr. J. Witte (Witte Klusbedrijf, Den Burg, The Netherlands)

The NIOZ ballast water team advisory committee consists of Prof. Dr. Hein de Baar (chemistry), Dr. J. van Bleijswijk (molecular biology), Dr. Corina Brussaard (microbial ecology) and Dr. Klaas Timmermans (head of the Department of Biological Oceanography at NIOZ).

The Quality Management Plan (QMP) and Quality Assurance Project Plan (QAPP) are reviewed annually by an external independent consultant. In 2012 this was performed by Dr. S. Kools of Grontmij (The Netherlands). In addition, at the request of the BSH a review on QA/QC aspects was performed by Dr. A. Cangelosi of GSI (USA). Comments of both parties have been used in the present project plan that includes both QMP and QAPP.

3. Quality system components

The quality system of the NIOZ BWTS test facility contains several components of which this QMP is the main document. The QMP is the joint responsibility of both project leaders of the NIOZ Ballast Water Project. The QMP forms the basis of the project's quality assurance and quality control (QA/QC).

The Quality Assurance Project Plan (QAPP) is the project-specific technical document reflecting the specifics of the NIOZ test facility, the BWTS tested, and other conditions affecting the actual design and implementation of the required experiments, such as test water quality, sampling and sample storing, the measurement of IMO-required variables and a general outline of the QA/QC. Variable-specific QA/QC measures are contained in each individual SOP, that together form section three of the QAPP.

Notebooks are used to record data, observations and deviations from SOPs, that are made during BWTS tests as well as during laboratory analyses. All notebooks are stored in the NIOZ Ballast Water archive. In a number of cases, such as in monitoring NIOZ harbour test water temperature and salinity, and in the in-line measurement of turbidity and salinity of test water at intake, data loggers are used to store information electronically. All digital

information is stored on a dedicated server that is accessible to Ballast Water Project personnel only.

Specific forms are used to record data during specific routine monitoring activities. These activities include monitoring of meteorological and environmental data and equipment functioning during BWTS tests, as well as the identification and enumeration of >50 µm organisms. All forms are stored on paper in the NIOZ Ballast Water archive or in electronic format on the Ballast Water Project server.

Specific forms for sample custody are also used in case of the analyses of pathogenic bacteria or specific chemical (toxicological) analyses, which are carried out in commercial laboratories of third parties.

Standard Operating Procedures or SOPs are available for each IMO variable that the test facility is obliged to measure. For abiotic variables the analysis methods stem from decades of NIOZ experience as an oceanographic institute. New methods, in particular those for biological variables, are or will be published in international peer-reviewed scientific journals, i.e. subjected to anonymous scientific quality control.

In addition, SOPs are present for specific actions during the BWTS testing such as for adjusting the salinity of the test water, flow cytometer data analysis or the cleaning of ballast water tanks. SOPs are numbered by year and version. Paper copies are kept in the NIOZ test facility archive. Electronic copies are stored on a dedicated server that is accessible to Ballast Water Project personnel only. SOPs are critically reviewed during the operation of the test facility as well as during annual reviews by the quality manager. SOPs relevant to the QAPP can be found in section 3 of this project plan.

The test protocol (QAPP, QMP and SOPs) are submitted before the start of the BWTS tests to the class societies that were designated by the national authorities. These are the BSH (Bundesamt für Seeschifffahrt und Hydrographie or Federal Maritime and Hydrographical Agency, Hamburg) for Germany and Lloyds Register (London, Rotterdam) for The Netherlands (Inspectie Leefomgeving en Transport, ILT), United Kingdom (Maritime and Coastguard Agency, MCA) and Greece. The BSH and Lloyd's Register are notified in advance of the BWTS test dates including the days of intake and discharge, and are invited to witness all test related activities at the NIOZ test facility. In 2012 additional oral presentations on BWTS testing at NIOZ were given to the Dutch IMO representative and ILT, the BSH and to Lloyd's Register.

4. Personnel qualifications and training

All research personnel of the NIOZ test facility are actively involved in the development of SOPs and the QA/QC measures as part of these SOPs. The quality manager is responsible for the QA/QC in each SOP and he checks the proper execution of QA/QC during BWTS testing and the analysis of samples and data. The quality manager will report the acquired QA/QC information to the project leader of ballast water research who will, if necessary, instruct the personnel to adjust QA/QC measures. The quality manager will also use this information in his evaluation of the BWTS test results.

All research personnel of the NIOZ test facility is trained in such a way that at least two staff members are able to perform a specific sampling or analysis. This should prevent personal bias in sample and data analysis. In addition, this procedure ensures that in case of unexpected staff absence, sampling or sample analysis can still be carried out by trained personnel.

In case of non-automated sample analysis, the microscopic identification and enumeration of planktonic organisms the research personnel is trained in groups of three. At the beginning of each year NIOZ test water is analysed by all three staff until the difference in individual plankton concentrations is <10%. Furthermore, in the case of >50 µm organisms, the correct execution of sample and analysis procedures and identification and enumeration of the organisms is audited annually by an external independent consultant. In 2012 this was performed by Mr. F. Fuhr of KiTe Aquatic Resources Consulting (The Netherlands).

Additional training of staff members takes place on-the-job during inter-comparison workshops of the ballast water test team in cooperation with the planktonlab of the Department of Biological Oceanography of NIOZ. One such workshop was the comparison

of flow cytometers held in January 2012. Furthermore, a statistics course is planned for autumn-winter 2012. A QA/QC course is planned for the winter of 2012-2013.

5. Procurement of items and activities

The BWTS test facility is part of the NIOZ research institute and is required to use the NIOZ facilities for procuring items and services that include all sampling and laboratory equipment and chemicals. The financial management assistant to the test facility ensures that all procedures are as required by internal NIOZ rules and by Dutch law. Material and equipment for the test installations is procured by Mr. A. Smit of Smittech (Den Hoorn, The Netherlands).

In all cases of procurement the quality, costs and supplier reputation are evaluated by research staff or Mr. A. Smit in collaboration with the project leader of Ballast Water Research or, in his absence, the quality manager.

Outsourced sample analyses are performed by commercial parties that have an established quality management program.

6. Documents and records

Documents related to the NIOZ test facility and BWTS testing include: legal and financial contracts, BWTS-specific project plans (including QAPP, QMP, SOPs), North Sea Ballast Water Opportunity (NSBWO) documents, notebooks, data and custody forms, student reports, internal reports on, for instance, inter-comparison workshops and scientific publications.

Legal and financial contracts are archived as required by NIOZ under the responsibility of the financial management assistant and the overall project manager. North Sea Ballast Water Opportunity (NSBWO) documents are maintained by the general and financial management assistants or by the project leaders. Notebooks, data and custody forms are archived for at least five years by the project leader of ballast water research. BWTS-specific project plans (including QAPP, QMP, SOPs), student reports, internal reports and scientific publications are also archived by the project leader of ballast water research.

Reports on new technologies as well as for bench-scale tests to examine prototypes and components of BWTSs and other documents pertaining to the test facility are numbered sequentially as "NIOZ Ballast Water Report yyyy-1,2,..".

The generic BWTS-specific project plan (including QAPP, QMP, SOPs) as well as the G8 and G9 reports will also be numbered sequentially as NIOZ Ballast Water Reports. These documents will be made publically available on the NIOZ web site.

7. Computer hardware and software

The computer hardware that is used in the NIOZ test facility consists of lap tops and personal computers that use Windows™ based software. Both hard- and software are maintained by the NIOZ "Information and Presentation Centre" (IPC). IPC is also responsible for the security of data and other information, for instance by using up-to-date virus protection and making daily back-ups. The NIOZ network can only be accessed by authorised personnel and students.

In addition, all BWTS test data are stored on a dedicated network-share that is accessible only to authorised ballast water team members. This authorisation needs to be granted by both IPC and one of the project leaders of the ballast water project.

All software used by the NIOZ test facility, including Microsoft Office and dedicated software for data analysis, is legally obtained.

8. Planning

Manufacturers of BWTSs that approach the NIOZ facility for testing of their equipment should be aware of several planning criteria.

First of all, because NIOZ uses natural test water, spring and summer are the only periods of the year in which proper testing according to IMO regulations and NIOZ quality objectives can be performed. Second, as part of a research institute it may be of interest to the facility that the BWTS to be tested is of a different design as previously tested BWTSs.

The two project leaders of the test facility jointly decide on the admission of a BWTS for land-based testing. In case of requests for combined land-based and ship-board tests the admission will be discussed with the NIOZ partners IMARES and GoConsult. Contracts on behalf of the NIOZ will be signed by one of the NIOZ directors.

The provisional project plan and planning for testing the BWTS is made by the project leader of ballast water research. Next, the provisional project plan and planning is discussed in the ballast water team where all practical issues including those related to quality management are addressed.

9. Implementation of work processes

Quality management objectives as specified in this QMP and in the QAPP and SOPs are implemented in the work process. At the lowest level, individual members of the facility's test team are responsible for carrying out quality checks as detailed in SOPs on a daily basis. The quality manager is responsible for supervising QA/QC activities during all test and laboratory activities. The quality manager will give his feedback during each team meeting, that is scheduled at least bi-weekly during BWTS tests. On advice of the quality manager, the project leader of ballast water research gives permission to revise quality documents or to produce new documents.

10. Assessment and response

During BWTS tests inspections may be made by inspectors of Lloyd's Register or the BSH depending on the national authority that will apply for type approval at IMO.

All ballast water team members are obliged to report deviations from quality standards or procedures to the quality manager. At the end of each BWTS test the quality manager will report on all deviations from the QAPP or from QA/QC measures in SOPs. The quality manager also checks the BWTS test data that are compiled by the project leader of ballast water research. The project leader of ballast water research will report any deviations in harbour test water monitoring data and intake test water data from the QAPP to the quality manager.

11. Quality improvement

All team members are motivated to enhance specific QA/QC measures and methods. Regular feedback between team members and the quality manager will enhance self-improvement. In addition, the NIOZ quality objectives are submitted to independent external reviewers.

NIOZ test facility methods are compared and discussed in NSBWO workshops with other test facilities and experts in the field of ballast water research. Proficiency tests for the enumeration of aquatic organisms will improve the quality of analyses and will enhance the comparison of results between test facilities.

The NIOZ test facility invites comments to improve the quality of its tests and its test data, for instance from class societies and through its membership in the Global Testnet.

Section 2: Quality Assurance Project Plan (QAPP)

1 INTRODUCTION

The QAPP detailed in this document forms the basis for the biological efficacy testing of the XXXXXXXX Ballast Water Treatment System (BWTS) manufactured by Xxxxxxxx GmbH (Kiel, Germany) in 2012 at the ballast water treatment facility of the Royal Netherlands Institute for Sea Research in The Netherlands. The first version of this protocol was submitted to the BSH (Bundesamt fuer Seeschifffahrt und Hydrographie or Federal Maritime and Hydrographical Agency, Hamburg, Germany) in March 2012 in advance of the land-based tests that started on April 12 of that year.

This Xxxxxxxx test protocol is a combination of a Quality Assurance Project Plan (QAPP) and a Quality Management Plan (QMP). In previous versions of this protocol these two were merged. The QAPP is a project specific technical document reflecting the specifics of the BWTS tested, the test facility, and other conditions affecting the actual design and implementation of the required experiments. The QMP should address the quality control management structure and policies of the test facility. In 2012 the combined QAPP and the QMP, collectively known as the quality control/quality assurance (QA/QC) program, was extensively extended when compared to previous years.

Nevertheless, the BSH invited dr. A. Cangelosi (Northeast-Midwest Institute, USA) to review the QA/QC section of the protocol. In addition, NIOZ commissioned a review of the entire protocol to dr. S. Kools (Grontmij, The Netherlands). The present version of the protocol is revised in reaction to these two reviews.

In the summer of 2012 the BSH decided that a sufficient number of marine (saline) tests had been performed earlier that year and that five successful freshwater tests were needed for type approval. This meant that additional information had to be added on freshwater sampling and testing in the relevant chapters of this protocol.

The tests outlined in this protocol will evaluate the biological efficacy as outlined in the Guidelines for Approval of Ballast Water Management Systems, G8, adopted on 22 July 2005 as Resolution MEPC.125(53) (IMO, 2005) and as adopted in revised form on 10 October 2008 as Resolution MEPC.174(58/23, Annex 4) of the Ballast Water Convention of the IMO (IMO 2008).

The test protocol will be submitted for Type Approval by the applicant (Xxxxxxxx GmbH) to the German National Administration (BSH).

The Xxxxxxxx BWTS is composed of two main modules to disinfect ballast water of ships. One module is a filtration system, the second a UV-reactor. Both are operated during ballast water intake. During de-ballast operations the ballast water from the tanks passes again through the UV-reactor before discharge.

The applicant gives a brief description of the main components of the BWT system in chapter 2.

A detailed description of the test facility and the design of the test, sampling, sample storage and descriptions of the measurements of abiotic as well as biological variables are given in ensuing chapters. Because NIOZ is an academic research institute additional methods to count organisms and to establish their viability are continuously being developed. These methods may be applicable to efficacy testing according to the present as to potentially future D-2 Ballast Water Performance Standards and G8-guidelines.

2 GENERAL DESCRIPTIONS

Figure 1. Aerial view of the NIOZ harbour (lower right), NIOZ and the TESO ferry connecting the island of Texel with the main land (top). The Mokbaai is the source for additional suspended solids. ©Photo: Simon Smit Photography, Den Burg, Texel.

2.1 NIOZ profile

All tests of the Xxxxxxxx BWTS will be carried out under supervision of the Royal Netherlands Institute for Sea Research, Landsdiep 4, 1797 SZ 't Horntje, Texel, The Netherlands (from here on NIOZ, for details see: www.nioz.nl)

NIOZ Royal Netherlands Institute for Sea Research is the National Oceanographic Institute of the Netherlands. NIOZ is an institute of the Netherlands Organization for Scientific Research (NWO). The institute employs about 340 people at locations on the island of Texel on the border of the North Sea and the Wadden Sea (main location) and in Yerseke in the southwest of the country. The annual budget is approximately €30 million.

The mission of NIOZ is to gain and communicate scientific knowledge on coastal seas and oceans for a better understanding of the system and sustainability of our planet, to manage the national facilities for sea research and to support research and education in the Netherlands and in Europe.

In order to fulfil its mission, the institute performs tasks in four specific fields.

Research: The emphasis is on innovative and independent fundamental research in continental seas and open oceans. Increasingly, the institute also carries out research based on societal issues. The senior scientists at NIOZ all participate in international research projects. Several of them also hold a professorship at Dutch or foreign Universities.
Education: The institute educates PhD students and master students of universities and schools for professional education. Together with several universities, NIOZ also organises courses for PhD students and master students in the marine sciences. A number of our senior scientists is also appointed as professor at Dutch and foreign universities.

Marine Technology: NIOZ has its own workshops for mechanical, instrumental en electronical engineering. Here, marine research equipment is being designed and built according to the wishes of our individual scientists.
Facilities: NIOZ invites marine scientists from Dutch and foreign institutes and universities to write scientific proposals involving the institute's research vessels, laboratories and large research equipment. Our ocean-going research vessel 'Pelagia' is shared on a European level in the 'Ocean Facilities Exchange Group' (www.ofeg.org)

The basic scientific disciplines at NIOZ are physics, chemistry, biology and geology. Multidisciplinary sea research is regarded as one of the main strengths of the institute. Therefore, the research is organised in 5 multi-disciplinary themes: 'Open ocean processes, Sea floor dynamics, Wadden and shelf sea systems, Climate variability and the sea and Biodiversity and ecosystem functioning'.

Together with a number of oceanographic partners, NIOZ also maintains the popular marine website www.seaonscreen.org.

For more information, please contact our Communication & PR department at cpr@nioz.nl, or visit our website at www.nioz.nl

NIOZ has extensive experiences in the field of ballast water and ballast water treatment technologies at its harbour on the island of Texel. During the past seven years several pilot test for ballast water treatment were conducted in the NIOZ harbour and so far, between 2007 and 2010 seven full scale land-based tests were carried out for Final and Type Approval.

2.2 Profile of the Xxxxxxxx Group

2.3 Technical Overview of Xxxxxxxx's BWTS

Xxxxxxxx has developed a 100% chemical free BWTS (Figure 2). It is based on the combination of filtration + UV treatment. There are no active substances needed for the treatment of the ballast water, or for the cleaning of the UV system. The system was designed with emphasis on retrofitting (e.g. installation of the different components as required by different engine room designs). However, skid-mounted systems will also be available, which are optimized for foot-print.

Xxxxxxxx's BWTS is a modular ballast water management system. The system is installed in bypass to the main ballast line and provides a safe, flexible and economical process for the treatment of ballast water and eradication of aquatic invasive species. Treatment of ballast water is achieved through a simple and efficient two-step process.

1. Filtration

The ballast water passes through an automatic back flushing filter capable of removing particulates, and organisms (zooplankton and phytoplankton) using a 40 µm super mesh screen. The screen is arranged in cone shaped filter candles, where the number of filter candles varies with the treatment related capacity (TRC) of the BWTS. During the NIOZ G8-tests to be performed at 200 m^3/h the number of filter candles will be nine.

The automatic cleaning cycle of the filter is activated by an increased pressure drop across the filter. The filter candles are cleaned one after the other without interruption of the filtration process. The frequency of the filter back flushes depends on the quality of the water (e.g. the TSS content). The concentrate is discharged over board. This ensures that the screen is kept clean and the filtration process maintained at maximum efficiency at all times.

2. UV disinfection

The filtered ballast water is directed into the disinfection chamber where a cross flow arrangement with two medium pressure ultraviolet lamps delivers the high intensity irradiation. The UV light intensity is continuously monitored during system operation so that intensity is maintained above pre-set values to ensure delivery of the required dose. The ultraviolet lamps are housed within quartz sleeves and an automatic mechanical cleaning system minimizes bio-fouling and controls the accumulation of deposits on the UV lamp sleeves.

Figure 2. The Xxxxxxxx ballast water treatment system to be tested at NIOZ in 2012. This design allows for an easy exchange of the filters.

At discharge, the automatic backwash filter is by-passed and the ballast water is pumped from the tanks through the UV disinfection chamber only. Thus the ballast water is subjected to UV disinfection treatment prior to discharge overboard.

Specific features/advantages of Xxxxxxxx's BWTS

The filtration step
Xxxxxxxx's BWTS can be operated with two different types of filters, giving it the maximum possible flexibility in addressing different installation requirements.

The UV-lamp system
Xxxxxxxx's BWTS uses special medium pressure UV lamps with a reduced Mercury content. These lamps are mounted to a solid flange and they are surrounded / protected by a sealed robust quartz sleeve. This complete UV-lamp system, containing two lamps, is fitted to the UV reactor by a few screws. Access to the UV reactor for maintenance is from a single side only.

The lamp recycling scheme
On an annual basis, the UV-lamp system should be send off to Xxxxxxxx for refurbishment. The renovated lamp system will be returned to the ship with a new 1 year operating guaranty, if the lamp system has not been installed for a total time of more than 2 years.

The automated cleaning system of the UV

This is the first NON-chemical in place cleaning system for UV-reactors (CIP-system). It uses rubber cleaning elements that are supplied on demand to the UV reactor after the BW operation is finished. The cleaning process is triggered by the intensity measurement of the individual UV lamps. Start, stop and duration of the cleaning process are controlled by Xxxxxxxx's BW system. No manual interference is needed.

Figure 3. Process plan of Xxxxxxxx's BWTS. Water flows: black lines: at intake; yellow lines at discharge and red lines during cleaning of the UV unit.

Calculation of the UV dose
The UV dose is calculated directly from the water quality (UV transmittance of the water) and from the flow rate. The use of single electronic ballast units for each individual UV lamp allows for a maximum of flexibility in the power consumption. The UV lamps are operated at the most suitable dose rate at any time, allowing for significant savings in overall power consumption of the system.

The UV dose at T10=70% will be about m³/h flow rate. This dose is above the requirements for a 4 log reduction of many microorganisms, if no photo repair mechanism occurs. As there is no light inside the ballast water tanks, our system accepts this dose during ballasting only. If the UV-T decreases further, the flow will be reduced

automatically to ensure that the minimum dose is maintained. During de-ballasting, the water is much clearer, i.e. UV-T is higher, and the Xxxxxxxx system will dose between J/m² and J/m², which is well above the dose requirements (for a 4 log reduction) for the majority of organisms, even in the presence of photo repair mechanisms.

Xxxxxxxx's BWTS will be tested at a treatment related capacity (TRC) of 200 m³/h during the land based Type Approval Tests at the NIOZ test facility. There will be two UV-reactors and two different filters involved during the tests. The general layout of the test system should be according to the P&ID in Figure 4.

Figure 4. Piping and Instrumentation diagram of Xxxxxxxx's BWTS for a TRC (treatment related capacity) of 200 m3/h.

3 TEST FACILITY

3.1 NIOZ test facility

The land-based tests will be carried out on the island of Texel (NIOZ harbour, NL) from March to July (spring and early summer season). The NIOZ test-site is equipped with two times (Navicula and Pelagia quay) three silos or underground storage tanks of 300 m³ each to simulate ship's ballast water tanks (Figure 5).

The NIOZ harbour is located at the Marsdiep tidal inlet between the North Sea and the Wadden Sea. By sampling in different phases of the tidal cycle, waters from different seas with different abiotic and biological characteristics can be used in G8-tests. The area as a whole is characterized by a rich and varying biodiversity and high number of various planktonic organisms, especially in the spring and early summer period. During the test cycles the numbers of organisms present in the water will continuously be monitored to assure that the validity of the test cycles is in accordance with § 2.3.20 of the G8-guidelines.

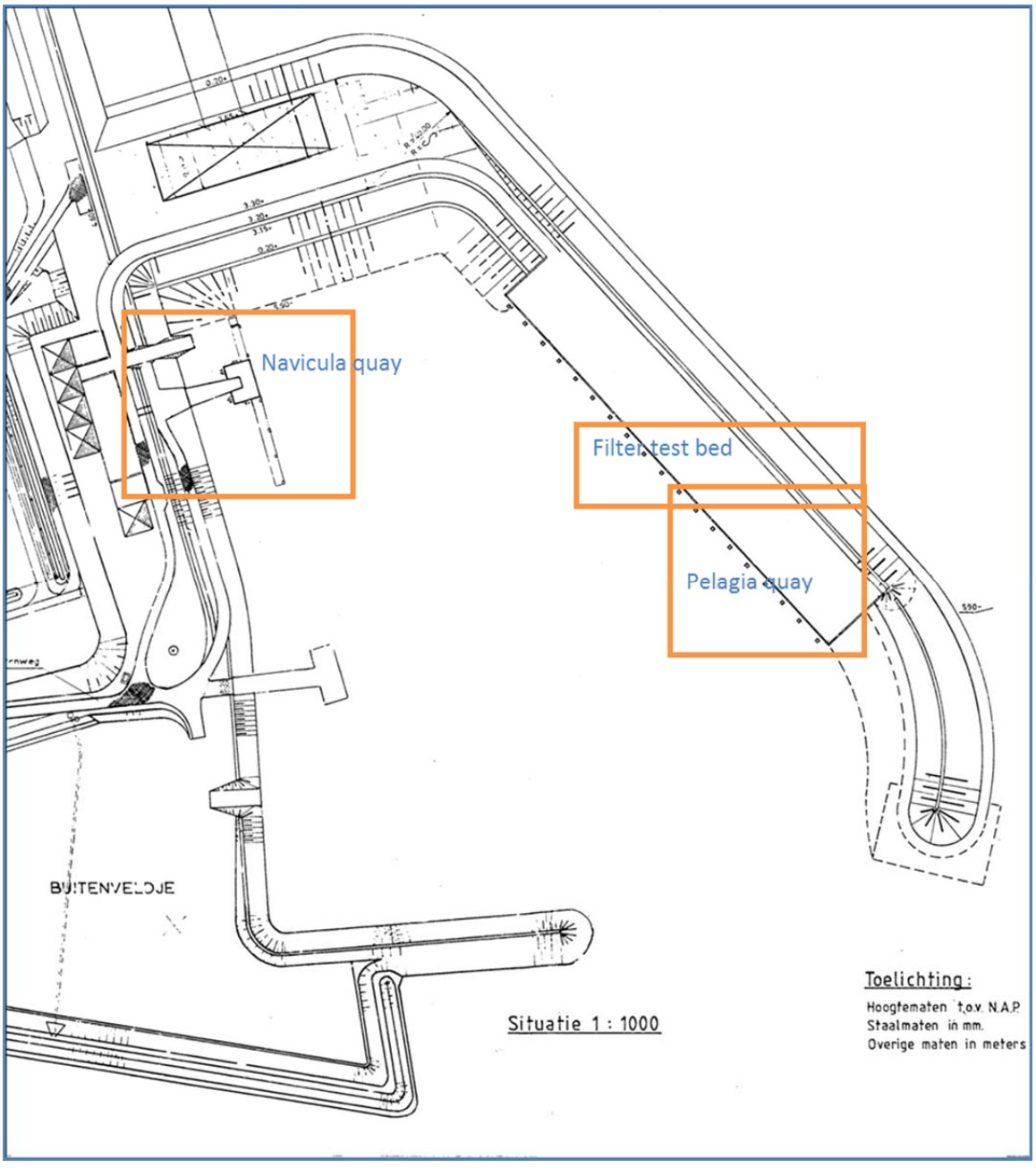

Figure 5. Schematic of the NIOZ harbour test facilities, including the two quays (Navicula and Pelagia) that are used in G8-tests.

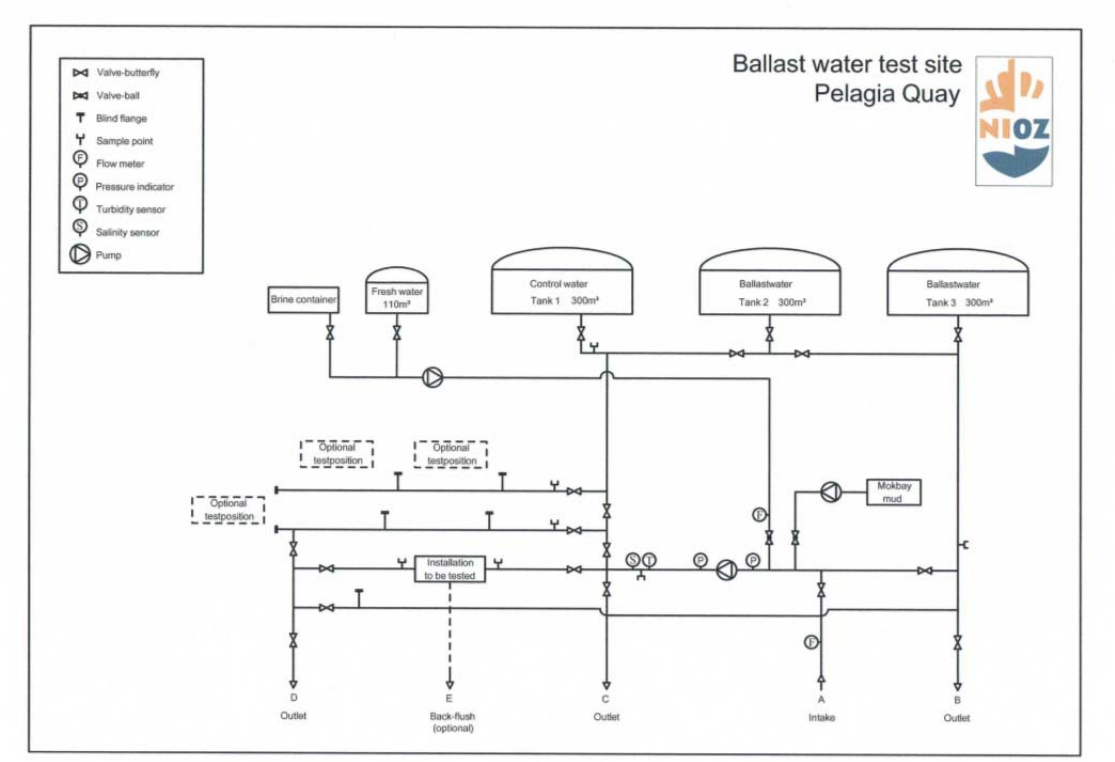

Figure 6. Piping and Instrumentation diagram of the Pelagia quay test site at the NIOZ harbour. The installation to be tested is a UV-treatment system. The installation consists of three ballast water tanks, one for control (untreated) water and two for treated water. Brine or freshwater can be added to adjust the salinity of the test water. Mud can be added to increase the concentration of Total Suspended Solids (TSS). Flow rates, system pressures, salinity and turbidity are monitored during intake and discharge. P&I diagrams are available for both Navicula and Pelagia quays and for different intake and discharge scenarios.

3.2 Freshwater intake in Den Helder and transport to NIOZ

Freshwater in Den Helder will be taken in at location B (Figure 7) by a ship that normally transports gravel and sand with a capacity of 650 m^3. The ship will be cleaned of any remaining sand before taking in water. The intake water will be pumped in the hold with tubes that are suspended at a height of maximally 1 meter below the water surface. Intake will take place on the afternoon prior to the tests.

The Den Helder harbour is monitored by NIOZ partner IMARES. According to IMARES Den Helder harbour is eutrophic and very productive. In May-July 2012 the concentration of >50 µm organisms was between 68,000 and 22,100,000 m^{-3}. For the 10-50 µm organisms this was 397 to 8,040 per mL. IMARES' experience at another location in The Netherlands learned that organism concentrations can remain high up till winter. Only when water temperature drops below ca. 7°C the zooplankton production will stop. Such low temperatures can be expected in November-December, but not during the planned G8-tests in September-October

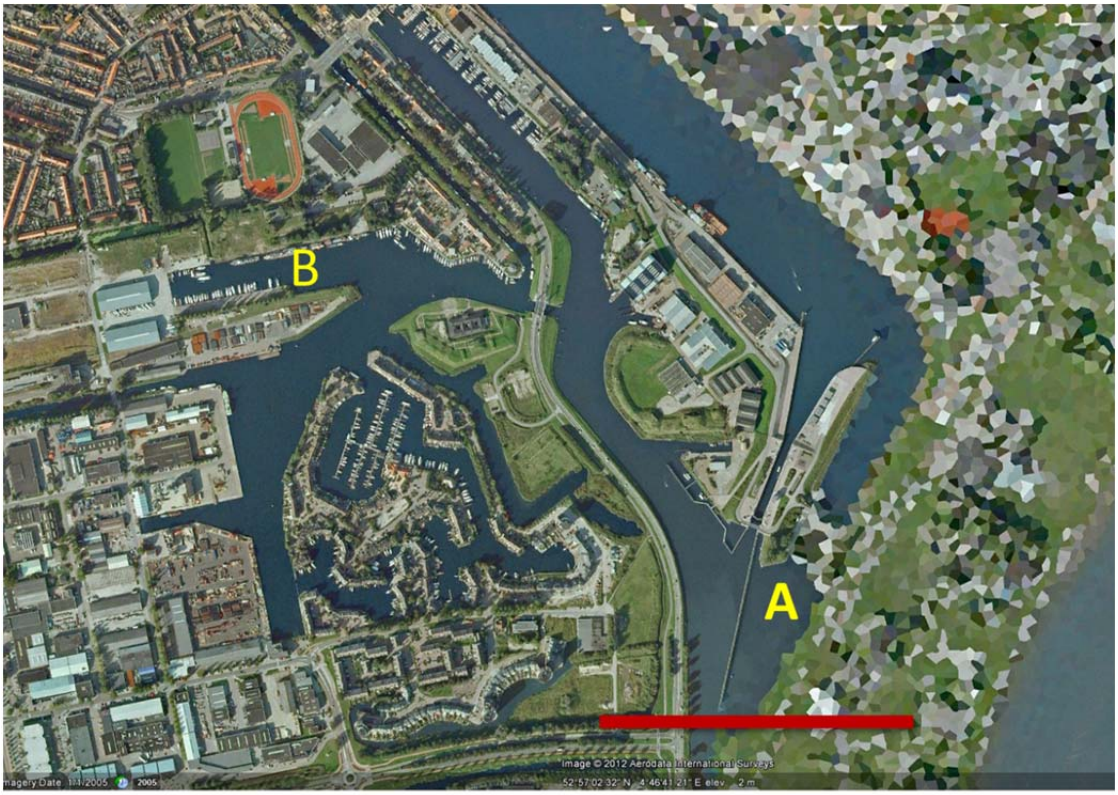

Figure 7. Freshwater intake at B („Spoorhaven"), the IMARES monitoring site in Den Helder harbour. The red horizontal bar is 0.5 km. Site A was the regular monitoring site; site B is the new IMARES monitoring site where freshwater for BWTS testing at NIOZ will be collected.

The freshwater test water is transported over a relatively short distance across the Marsdiep tidal inlet of the Wadden Sea within 12 hours by ship to the NIOZ harbour (Figure 8). The day following intake at Den Helder, i.e. within 24 hours, the test water is available in the NIOZ harbour and ready to be pumped into the NIOZ harbour installation.

Figure 8. The test water will be transported to the NIOZ harbour over a distance of 6.5 km in less than 12 hours.

The pump to be used is an Amarex KRTK 100-401/354WG-S that will be suspended by a crane from the NIOZ Pelagia quay (Figure 9). The crane will be able to steadily position the pump because the freshwater surface level will change according to deballasting and tide. This pump will be connected to the NIOZ installation (Figure 6) by a flexible hose.

Figure 9. The Amarex pump to be used in pumping freshwater from a boat into the NIOZ installation.

3.3 BWTS test set-up: treatment and control tanks

A typical test of a treatment system is performed with two treatment tanks and one control tank that are filled in rapid succession, i.e. on the same day at approximately the same time in the tidal cycle (Figure 6). The control tank with untreated water serves as reference to examine the effect of the treatment, including holding for at least 5 days (§2.3.35 G8-guidelines). The control tank can also indicate an unexpected source of mortality due to the testing arrangement (§2.3.37 G8-guidelines). Therefore, the average discharge results in the control water should not be less than or equal to 10 times the values in regulation D-2.1 (§2.3.36 G8-guidelines).

3.4 Toolbox meetings

The general test set-up is described in Figure 6 and in §3.2. Directly prior to each test a tool box meeting is held on the quay site with all team members. During this meeting all actions, such as order of tanks to be filled and flow rates, are briefly discussed.

3.5 Ballast water book

All manufacturers should log their activities in a ballast water book issued by the NIOZ. Several books may be issued during the entire test period. These books remain at NIOZ in the appropriate dossier.

3.6 Test season: time planning

The intermediate and high salinity range test season at the NIOZ harbour is restricted to spring and summer. In this period of the year sufficiently high numbers of organisms are naturally present in the North and Wadden Sea. At NIOZ the test water is not enriched with organisms, neither artificially cultured nor collected at sea. In general, early spring sea water has a lower salinity then in summer due a decrease in river discharge. A decrease in wind speed during spring will lead to diminishing concentrations of total suspended solids (TSS) and, therefore, sediment from the nearby Mokbaai (Figure 1) is added to increase TSS to the required value of 50 mg/l for brackish water.

The freshwater test season, according to IMARES data, begins in March and probably ends in November-December.

In February sampling and measurements in the harbour start in order to monitor the start of the spring plankton bloom. In March the first G8-tests may be performed, depending on natural circumstances such as water temperature and underwater light climate that affect the plankton development.

The first set of tests is carried out at the intermediate salinity range of G8 (§2.3.17) because in early spring the freshwater content of the Wadden Sea is relatively high. Test water is pumped from the harbour at low tide when low salinity Wadden Sea water flows towards the North Sea. The second set of high salinity tests is performed in late spring or early summer. Test water is pumped from the harbour at high tide when relatively saline North Sea water flows towards the Wadden Sea.

After consultation with the BSH, the Xxxxxxxx BWTS tests carried out in spring and early summer of 2012 were combined to one salinity (saline) range. This meant that an additional series of freshwater tests had to be performed. The first appropriate months for these freshwater tests were September and October.

4 TEST WATER: QUALITY, SAMPLING, STORING

4.1 Abiotic quality

The land-based test cycles have to be carried out at specific water qualities as defined in the G8-guidelines. The NIOZ-harbour represents a brackish water environment with a varying salinity (20 – 35 PSU). High salinity water originating from the North Sea is taken in around high tide. Low salinity water from the Wadden Sea is taken in around low tide. The salinity of the Wadden Sea water is dependent on the discharge of freshwater from Lake IJssel, which itself depends on the amount of rainfall and on the flow rates in the rivers Rhine and IJssel. In an effort to maintain a minimum 10 PSU salinity difference as requested under § 2.3.17 of G8, per tank 15 m^3 freshwater is added in the pipelines to the natural water prior to the pump to reduce the ambient salinity (ca. -2 PSU) and 8 m^3 brine (100 kg m^{-3} industrial quality salt) is added to increase salinity (ca. + 2 PSU) at the second set of test series. At present only brackish and high salinity seawater conditions can be tested at NIOZ.

Figure 10. Extra natural sediment from the "Mokbaai" will be added when required to meet the minimum TSS concentration for the given salinity test cycle.

In addition, per ballast tank 20 litre of mud (15.6 kg dry weight) from the nearby Mokbaai (Figure 1) will be added to the low salinity tests in order to reach the required TSS value of >50 mg/L (Figure 10). The organic carbon concentration is important in testing systems that use oxidizing agents as active substances. DOC concentrations are usually below 5 mg/L in low salinity test water but no DOC additions are made because the high POC values (>10 mg/L) in the NIOZ test water are considered to compensate for that. In other words, the total organic load in low salinity test water is sufficiently high.

An overview of the required water quality, with respect to the composition of total suspended solids, particulate organic carbon and dissolved organic carbon is given in Table 1.

Table 1. Requirements of salinity, TSS, POC and DOC in the test water for the high salinity and intermediate salinity and freshwater test regimes according to the G8-guidelines.

	High salinity	Intermediate salinity	Freshwater	units
Salinity	> 32*	3 – 32*	<3	PSU
Total Suspended Solids (TSS)	> 1	> 50**	> 50**	mg/L
Particulate Organic Carbon (POC)	> 1	> 5**	> 5**	mg/L
Dissolved Organic Carbon (DOC)	> 1	> 5	> 5	mg/L

*to obtain a 10 unit salinity difference either brine or fresh water may be added
**natural mud is added to increase TSS as needed, this also increases POC

4.2 Biological quality

In order to establish the biological efficacy of the BWTS it should be tested with water containing a high concentration of organisms as well as a sufficient biodiversity (§ 2.3.20 of G8). This is required by G8 to guarantee the effectiveness of the BWTS in different ecosystems across the globe. The variety of organisms in the influent test water should be documented according to the size classes mentioned in Table 2.

Natural water, originating from the coastal zone of the North Sea (high tide) and the inner Western Wadden Sea (low tide) will be used. The test period will cover the whole spring and early summer of the plankton growth season and therefore includes the natural occurring biodiversity and species succession. The ambient plankton content in terms of species diversity in the relevant size classes is very high. For instance in 2011 16 phyla and more than 70 species were detected during the test season (Table 3). Only 5 species and 3 phyla are required (§ 2.3.20 of G8).

Table 2. Minimal numbers and species diversity required at intake for different size classes and groups of organisms. 1 µm = 1 micron = 0.001 mm.

Intake test water		
Organism	unit	Variety
≥50 µm	> 10^5/ m^3	at least 5 species from at least 3 different phyla/divisions
≥10 and <50 µm	> 10^3/ mL	at least 5 species from at least 3 different phyla/divisions
heterotrophic bacteria	> 10^4/ mL	not further defined

The natural waters of the test area include a large range of organisms varying in sensitivity to mechanical stress, UV radiation or various active substances. Besides fragile organisms also plankton that is highly adapted to harsh environmental conditions, mostly hard shell organisms, are present in the test water.

For completeness, the plankton fraction <10 µm is also included in the NIOZ analyses although this is not required by the G8-guideline.

Table 3. Biodiversity as number of species in NIOZ test water according to phylum and to size class based on data from the 2011 spring and early summer test season. The test water contains at least 18 phyla with a total of 16 phyla in the 10-50 and >50 µm size classes. The total number of species in each G8-size class is 42 (8 phyla) for the 10≤µm<50, and 31 (10 phyla) for the ≥50µm size classes. Organisms <10µm that are not bacteria are not part of the D-2 regulation. The high test water biodiversity largely exceeds the G8-guideline (§2.3.20).

Phylum[a]	Number <10 µm	Number 10-50 µm	Number >50 µm
Amoebozoa		1	
Annelida			5
Arthropoda			13
Cercozoa		1	
Chlorophyta[b]	1	1	
Choanozoa	1		
Ciliophora		3	
Cnidaria			2
Cryptophyta	1		
Ctenophora			1
Echinodermata			1
Euglenozoa		1	
Haptophyta	3		
Mollusca			4
Myzozoa	2	6	1
Nematoda			1
Ochrophyta	12	28	2
Rotifera			1
Unknown[c]	4	1	
Total	**24**	**42**	**31**

[a] The taxonomic system is as follows: Kingdom (Archea, Bacteria, Animalia, Chromista ("Algae"), Plantae) – Subkingdom – Infrakingdom – Phylum – Subphylum - Division – Class – Subclass –Superorder – Order – Family – Genus – Species
[b] Division (no phylum for this group)
[c] The phylum 'unknown' contains several species of unidentified phytoplankton flagellates

4.3 General sampling strategy

Samples are generally taken:

1) In the harbour to assess test water quality before the pump. Harbour water samples are analysed regularly from February onwards in order to monitor the spring plankton bloom.
2) Immediately before the treatment equipment from the main pipeline but after the ballast pump that is used to pump up the test water from the harbour (control, T0),
3) Immediately after treatment from the main pipeline (treated, T0) and
4) During discharge from the main pipeline, after the pump, after 5 days (control and treated, T5) holding time (§ 2.3.2 and 2.3.26 G8-guidelines) and after completing a second passage through the BWTS when this step forms part of the treatment prescribed by the vendor of the BWTS, i.e. in the case of the Xxxxxxxx BWTS (treated only).

During ballast water tests samples will be taken sequentially, covering the entire intake or discharge periods.

During the tests the following sample sizes will be used:

1) Untreated water (control, T_0 and T_5): Sampling is conducted in-line, three times, with sample volumes of 20 L (>50 µm), 10 L and 2x 1 L. The 10 L samples are used to subsample for abiotic variables. The 1 L samples are used (1) to subsample for phytoplankton, bacteria, as well as for phytoplankton <10 µm and (2) microzooplankton (10≤µm<50). An additional 10 L sample is taken for an incubation experiment (T_0 only).
2) Treated water (intake, T_0): Sampling is conducted in-line, three times, with sample volumes of 1 m^3 (>50 µm) using 3 IBCs of 1000 L each. Furthermore, in-line sampling is conducted three times with a sample volume of 10 L and 2x 1 L. The 10 L samples are used to subsample for abiotic variables. The 1 L samples are used (1) to subsample for phytoplankton, bacteria, as well as for phytoplankton <10 µm and (2) microzooplankton (10≤µm<50). Additional samples of 10 L are taken for incubation experiments.
3) Treated (discharge, ≥T_5): Sampling is conducted in-line, three times, with sample volumes of 1 m^3 (>50 µm) using 3 IBCs of 1000 L each. Furthermore, in-line sampling is conducted three times with a sample volume of 10 L and 2x 1 L. The 10 L samples are used to subsample for abiotic variables. The 1 L samples are used (1) to subsample for phytoplankton, bacteria, as well as for phytoplankton <10 µm and (2) microzooplankton (10≤µm<50). Additional samples of 10 L each are taken for incubation experiments.

The (sub)-sample volumes taken from the in-line samples deviate from §2.3.32-33 (G8-guideline): 10≤µm<50: 1 L for untreated water and 10 L for treated water; bacteria: 0.5 L. Note that G8 only specifies sample sizes, but not which volume of these samples should actually be analysed. The reason for this deviation is that sample volumes of 10 L for organisms 10≤µm<50 and 0.5 L for heterotrophic bacteria are impractical: much smaller volumes will eventually be analysed without compromising the analytical accuracy. The sample volumes for pathogenic bacteria are 300 and 600 mL.

4.4 Abiotic variables: sampling and storing

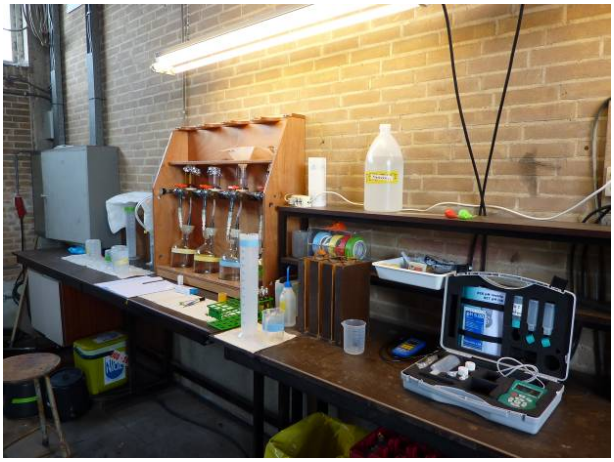

Figure 11. Navicula quay laboratory for first line handling of samples.

The measurement of the following abiotic (environmental) variables is required during sampling (G8 § 2.3.25):

1) salinity,
2) temperature,
3) Particulate Organic Carbon (POC),
4) Total Suspended Solids (TSS),

5) pH,
6) Dissolved Oxygen (DO) and
7) Dissolved Organic Carbon (DOC).

Turbidity is also mentioned in § 2.3.25 (G8) but experience has learned that it is difficult to reliably measure this variable in water samples of limited volume taken during intake or discharge. Furthermore, turbidity is a function of TSS, a variable that is measured accurately and with far better precision. In addition, NIOZ research has shown that at low TSS values a standard turbidity instrument underestimates the actual solid content of the water.

Rapid measurement and processing of samples for the basic variables is ensured by the NIOZ harbour laboratory on the Navicula and Pelagia quays of the test facility (Figure 11). A number of measurements is performed after sampling (e.g. temperature, salinity and pH), while the other samples (DOC, POC, TSS, DO) is processed for later analysis.

DOC samples are filtered through GF/C filters and sealed in pre-combusted glass ampoules or special screw cap bottles after adding hydrochloric acid (HCl). Sealed ampoules or bottles are stored at 4°C until analysis.

For TSS/POC measurements pre-weighed glass-fibre filters (GF/C) are used. Each filter is coded and stored individually in a Petri dish. The filtered water volume is not constant but depends on the particle concentration of the test water. The higher the particle concentration in the sample, the smaller the volume that can be filtered before clogging. Practical volumes are 100-1000 mL per sample. After filtration the filter is rinsed with demineralised water to remove sea salts. The filter is then put back in its original Petri dish and stored temporarily in a freezer at –20°C or long-term at -80°C until further analysis.

Glass Winkler bottles for oxygen (DO) are flushed at least three times their volume (ca. 120 mL) with test or control water. Great care is taken to avoid gas bubbles on the wall of the bottle or excessive mixing of air and water. Next, $MnCl_2$ and NaOH solutions are added to the bottle just below the surface by using dispenser units to fix the oxygen concentration. A stopper secured with a rubber band is put on the bottle and the bottle is gently mixed. Bottles are stored in a dark container filled with water of the same temperature as the samples until further analysis in the laboratory (Figure 12).

Figure 12. Glass bottles for Dissolved Oxygen (DO) measurements are stored submerged prior to analysis.

Figure 13. Sampling point on a tank at the Navicula quay for sampling dissolved gasses such as dissolved oxygen.

In special cases gastight tubing which is fitted to the sampling tubing is used to avoid exchange of gasses, such as CO_2 or oxygen, between water and surrounding atmosphere (Figure 13).

All environmental variables are measured in triplicate samples (§ 2.3.29 G8-guidelines).

From 2012 onwards, additional continuous measurements of temperature, salinity, flow rate, system pressure and turbidity will be made at intake and discharge by sampling via a specific sample port equipped with the appropriate monitoring instrumentation (e.g. S and T in Figure 3). The data will be logged electronically and will be used to monitor basic system variables during intake and discharge of the BWTS tests.

4.5 Biological variables: sampling and storing

Biological variables that need to be considered (Regulation D-2 and § 2.3.20-21 G8-guidelines) are:

(1) Concentration and diversity of organisms ≥50 µm,

(2) Concentration and diversity of organisms 10≤µm<50,

(3) Concentration of heterotrophic bacteria,

(4) Concentration of coliform bacteria,

(5) Concentration of *Enterococcus* bacteria, and

(6) Concentration of *Vibrio cholera*.

The groups of organisms ≥50 µm and 10≤µm<50 consist of both algae (phytoplankton, mainly < 50 µm) and animals (zooplankton, mainly >50 µm). According to the Ballast Water Performance Standard (Regulation D-2 and § 4.7 G8-guidelines) only viable organisms must be counted at the end of the test in both the treatment and the control tanks. For both of the IMO relevant size classes (organisms ≥50 µm and 10≤µm<50), multiple methods of enumeration and of assessing the viability are applied at NIOZ to verify the results at a high level of confidence. In principle all methods should give a conclusive answer with respect to numbers and/or viability of the (remaining) organisms.

At NIOZ the viability of all organisms is not only measured at discharge but during intake as well. Furthermore, the concentration of phytoplankton <10 µm is measured. All biological variables are measured in triplicate samples (§ 2.3.29 G8-guidelines).

4.5.1 Sampling organisms ≥50 µm

The samples of the untreated water (20 L) are taken in triplicate, collected in clean buckets that are filled directly for volumetric measurements, and poured through a 50 µm sieve made from the same gauze as the sampling nets used for the 1 m^3 IBC samples.

Discharge water samples are collected using Hydrobios™ 50 µm diagonal mesh size nets, as recommended in MEPC 54/Inf.3, that are fitted into 1m^3 IBC's (Figure 14). Sampling is

conducted via flexible hoses which are connected to the sampling points. To sample treated water the hose is put into the Hydrobios™ net. The whole sampling procedure will be timed in a way to cover the whole period of filling the ballast water tank with ca. 250 m^3 water. For practical reasons all organisms quantitatively retained on the 50 μm net are considered as larger than 50 μm in minimum dimension.

The organisms retained in the cod-end of the Hydrobios™ net are flushed into a beaker using a squeeze bottle containing filtered seawater. Organisms are kept in approximately 100 to 200 mL of filtered (≤0.2 μm filter) sterile water of the relevant salinity. Samples are transferred to the lab directly after sampling, treated for 2 hours with the viability stain 'Neutral Red', and counted. These samples are not stored.

Figure 14. Intermediate Bulk Containers (IBCs) of 1 m^3 each. A 50 μm Hydrobios™ plankton net is fitted in the containers through the lid for easy sampling**.**

4.5.2 Sampling and storage of 10≤μm<50 organisms

Samples for the 10≤μm<50 fraction are taken as undisturbed (unfiltered) one litre samples. This size-fraction is not separated from the organisms <10 μm or >50 μm at sampling or during sample processing but during data analysis. This approach reduces damage to more delicate organisms as ciliates and non-armoured flagellates.

Whole, intact samples of 1 L are stored in the dark prior to analysis in the laboratory. The total concentration of 10≤μm<50 phytoplankton cells is measured in non-fixed samples. The concentration of dead phytoplankton cells is measured using SYTOX-Green, also in non-fixed samples. The difference between the concentrations of total and dead organisms is the concentration of viable organisms.

As a reserve a 5 mL sample is fixed with formaldehyde and stored at -80°C for up to one year. This fixed sample can no longer be used to determine the viability of the organisms.

The samples for 10≤μm<50 microzooplankton are fixed with Lugol's solution; they can be stored in a refrigerator for up to one year before counting takes place. The viability of the organisms is assessed from their intact morphology.

4.5.3 Sampling and storage of total and viable heterotrophic bacteria

To determine the total concentration of bacteria samples fixed with formaldehyde are frozen and stored at -80 °C until counts are made after staining with PicoGreen. Dead bacteria in non-fixed samples are measured in fresh test water using SYTOX-Green; these samples cannot be stored. The difference between the concentrations of total and dead bacteria is the concentration of viable bacteria.

4.5.4 Sampling of human pathogens

Live samples for microbiological analysis are taken in sterile bottles of 300 or 600 mL and sent to a special laboratory (Eurofins/ C.mark) for further analysis. The samples are transported immediately after sampling using a cooled transport container (4 °C).

4.5.5 Sampling for additional incubation (viability) experiments

Additional incubation experiments, which are not required by G8, serve to better assess the effect of the different ballast water treatment systems and the five day tank storage on cell viability and concentration. In case the treatment is insufficient and residual viable organisms remain present or resting stages or cysts germinate, growth of the plankton will be stimulated under favourable conditions. This incubation method also allows studies on the effect of the treatment over a period longer than 5 days (up to 20 days).

The experiments usually involve control and treated water that were sampled at T5, the day of discharge. They are performed in 10 L flasks in a climate room under optimal growth conditions for the plankton community, including irradiance, temperature and turbulence. A sufficiently high level of nutrients is ensured through the addition of nitrate, phosphate and silicate, favouring phytoplankton growth and stimulating cyst germination throughout these experiments. The standard variables measured include phytoplankton and microzooplankton abundance (<10µm and 10≤µm<50), the viability of phytoplankton (PAM fluorimetry) and the abundance of bacteria. Usually the incubation time is seven days. In more elaborate experiments samples are taken daily and may include multiple analyses of phytoplankton and heterotrophic bacteria.

5 MEASUREMENT OF VARIABLES

A list of all abiotic and biotic variables that are required by G8 is provided in Table 5.1. All variables are measured in samples of test water, taken during intake and discharge as required. Turbidity is a function of TSS, and because turbidity is difficult to measure reliably at relatively low TSS concentrations, this variable is only measured in-line and not in separate samples. *Vibrio cholerae* is not present in NIOZ test water, cannot be added, and hence is not sampled for. An additional measurement is that of phytoplankton <10 µm, which is not an IMO requirement (Table 5.1).

All measurements are described in the Standard Operating Procedures (SOPs) that are listed in section 3 of this project plan. Samples for E. coli and enterococci are outsourced and analysed according to NEN-ISO standards (Table 5.1). A brief description of all relevant methods is given in the following paragraphs.

5.1 Abiotic variables

5.1.1 Salinity, Temperature and pH

Water samples for salinity, temperature and pH are collected in 10 L buckets. Measurements are either done immediately or after storage (maximum 6 hours) in the dark and at ambient temperature. Salinity is measured with a digital conductivity meter. Temperature is measured with a calibrated digital thermometer. pH is measured with a calibrated digital pH meter.

5.1.2 TSS/POC (Total Suspended Solids/Particulate Organic Carbon)

For TSS analysis filters are dried at 60°C for at least 8 hours and weighed again. The concentration of TSS per litre can be calculated from the sample volume and the weight difference of the filter before and after sampling. TSS is expressed as mg/L.

Next, to determine the POC concentration the same filter is combusted overnight at 500°C and allowed to cool in a dessicator and weighed again. The POC is calculated from the weight difference between this measurement and the dry TSS weight. POC is expressed as mg C/L.

5.1.3 Dissolved Oxygen (DO)

Fixed samples in Winkler bottles are acidified with H_2SO_4 prior to measuring the optical density (OD) at 456 nm with a spectrophotometer. The oxygen concentration is calculated using standards and expressed as µM O_2/L (or mg O_2/L = µM O_2 * 0.032). Since both salinity and temperature change over the season the oxygen concentrations is expressed as percentage relative to the natural saturation value for the given temperature and salinity.

5.1.4 Dissolved Organic Carbon (DOC)

The DOC concentration is determined in the laboratory by a high temperature combustion method using a Shimadzu TOC-Vcpn analyser according to Reinthaler & Herndl (2005). Standards are prepared with potassium hydrogen phthalate (Nacalao Tesque, Inc, Kioto, Japan). The mean concentration of triplicate injections of each sample (three in total) is calculated. The average analytical precision of the instrument is <3 %.

Table 4. List of variables measured in land-based tests at NIOZ

Variable	unit	IMO required	Reference
Salinity	PSU	Y	SOP
Temperature	°C	Y	SOP
pH	-	Y	SOP
TSS	mg/L	Y	SOP
Particulate Organic Carbon	mg/L	Y	SOP
Dissolved Oxygen	% saturation	Y	SOP
Dissolved Organic Carbon	mg/L	Y	SOP
Viable organisms ≥50 µm, including diversity	number per m^3, number of phyla and species	Y	SOP
Organisms 10-50 µm (phytoplankton)	number per mL	Y	SOP
Phytoplankton diversity	number of phyla and species	Y	SOP
Phytoplankton viability (PAM fluorimetry)	Fv/Fm	Y	SOP
Phytoplankton viability (SYTOX Green)	number per mL	Y	SOP
Organisms 10-50 µm (microzooplankton)	number per mL	Y	SOP
Microzooplankton diversity	number of phyla and species	Y	SOP
Microzooplankton viability	+ or -	Y	SOP
Organisms <10 µm (phytoplankton)	number per mL	N	SOP
Heterotrophic bacteria	number per mL	Y	SOP
E. coli	cfu per 100 mL	Y	NEN-EN-ISO 9308-1
Enterococci	cfu per 100 mL	Y	NEN-EN-ISO 7899-2

5.2 Biological variables

5.2.1 Counting of organisms ≥50 µm

For minimum dimension measurements the "body" of the organism should be measured, i.e. not antennae, tails etc. Examples are presented in Figure 15.

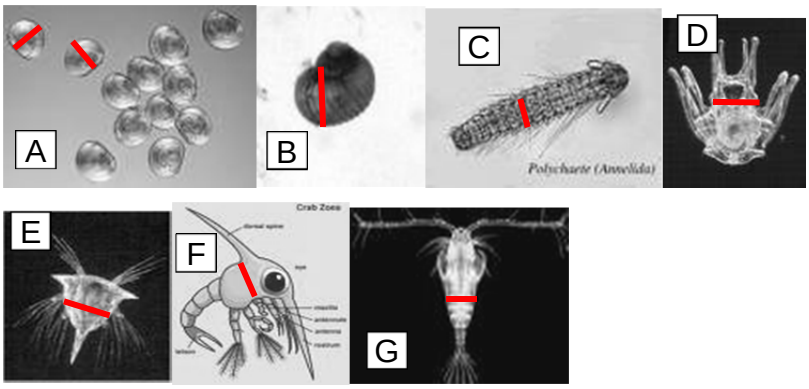

Figure 15. Minimum dimension measurements (red line) in selected organism types: A = bivalve larvae, B = gastropod larvae, C = worm, D = echinodermata larvae, E and F = crustacean larvae and G = copepod.

The viability of the organisms is assessed with Neutral Red, which stains living organisms only and does not affect their survival rate. This viability assessment remains unaffected by the possible death of organisms during staining or during sample analysis due to, for instance, warming of the sample. This is because organisms that die after addition of the Neutral Red will still be clearly stained, while those already dead prior to the addition will not be stained.

Neutral Red is pipetted in a ratio that yields an end concentration of approx. 1:50.000. The Neutral Red stock solution is 1:2.000, i.e. approximately 4 mL of stock solution is needed to stain a sample of 100 mL. The staining time is ≥ 2 hours. Stained samples are filtered over a 30 µm sieve and flushed into a Bogorov-dish with filtered seawater. Samples are analysed using a binocular with a 20x magnification for counting and up to 80x for species identification and measurements when necessary.

Neutral Red stains all major plankton groups, including phytoplankton, but it seems to have some limitations for bivalve larvae. For the latter movement, including that of hart and gill is used to verify viability. This is dependent on the expertise of the person analysing the samples. Therefore, only persons with a dedicated training period will analyse samples. Organisms that are able to swim are also considered alive. In doubt, the organism can be poked with a dissection needle. The procedure is outlined in Figure 16.

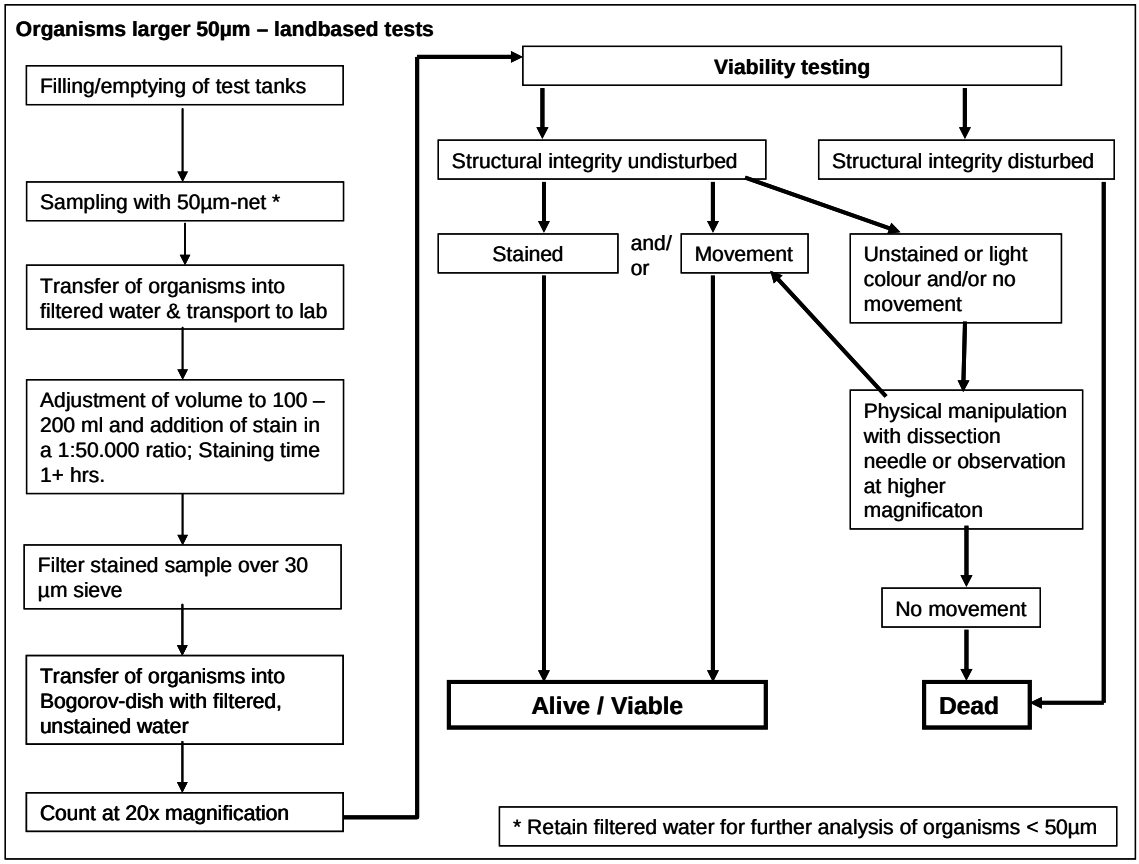

Figure 16. Sampling and viability assessment for organisms larger than 50 µm during land-based tests.

5.2.2 Counting organisms 10≤µm<50

5.2.2.1 Counting viable microzooplankton

The sample bottles are transferred to the laboratory and left undisturbed in the dark for all organisms and material to settle. After at least 24 hours the full bottle weight is measured. Using a water-jet pump and specialized tubing, the supernatant is aspirated. The bottle is weighed once more. The concentrate is transferred to small storage bottles that can be stored refrigerated for one year. The bottle is weighed once more to determine the sample and concentrate volumes.

The samples are analyzed with an inverted microscope at 200x magnification (method by Utermöhl). The fixed samples (or sub-samples thereof) are transferred into settling chambers and neutralized using sodium thiosulfate. After this, the sample is stained using Bengal rose stain. This stain specifically stains organic material and helps to identify organisms between sediment particles. After staining the samples are left undisturbed in the dark to settle. The time of settling depends on the settling chamber used, but should be at least 1 hour per cm height of the settling chamber. The iodide component of the Lugol's solution increases the weight of particles. Therefore they settle on the bottom-glass and can be counted. Live-dead-separation in these samples is mainly based on the structural integrity of organisms. This method can be applied for both zoo- and phytoplankton. Nevertheless certain groups are known to be affected by this standard method of fixation and therefore they will be systematically underestimated, if present.

5.2.2.2. Counting viable phytoplankton

Organisms in the 10≤µm<50 size class will be analyzed via flow cytometry (Figure 17), a semi-automated method used in the NIOZ for the counting of phytoplankton, bacteria and viruses. In principle, flow cytometry allows to assess a complete view of the effectiveness of the applied treatment technique for all organisms, irrespective of their size (Veldhuis & Kraay, 2000). By applying special staining techniques the numerical abundance of heterotrophic as well as autotrophic bacteria can also be estimated. The vitality of the

different organisms present will be addressed by using a specific fluorescent dye method (Veldhuis et al. 2001, Cassoti et al. 2005, Veldhuis et al. 2006, Peperzak & Brussaard 2011). In addition, the viability will be assessed by incubating discharge water (see § 4.5.5). The diversity of the phytoplankton is measured by microscopy of Lugol-fixed samples of the NIOZ test water by Koeman & Bijkerk (The Netherlands). This company is actively involved in international quality assessments (http://www.planktonforum.eu/)

Three replicate samples from both control and treated water are placed in the carousel of a bench top flow cytometer (Beckman Coulter XL-MCL or Becton Dickson Canto II), of which the Canto flow cytometer has an elaborate quality control system. All procedures and handling are conducted according to standard procedures (e.g. Shapiro 2003).

Samples will be counted using standard protocols covering the particles in the size range of ca. 2 to 50 µm. Total analysis time will be equal to an exact sampling volume of 1 mL or otherwise when relevant. Of all particles present in the volume counted, the cell size and the presence or absence of chlorophyll-*a* fluorescence will be measured. Only phytoplankton has chlorophyll-*a* fluorescence (Figure 18a,b).

Absolute numbers, cell sizes and chlorophyll-*a* content of the particles will be analyzed using the software package FCS Express V3 or V4 (DeNovo, US). Cell sizes will be estimated relative to 10 µm standard fluorescent beads (Flow-Check Fluorospheres, Beckman Coulter #660539) or relative to 10 and 50 µm beads on the Canto II.

For measuring viable phytoplankton, three subsamples will be stained with SYTOX Green (Veldhuis et al, 2001). This nucleic acid specific dye only stains DNA of cells with a compromised cell membrane. Of each phytoplankton cell present the green SYTOX fluorescence (Figure 15b) will be determined and compared with the green autofluorescent signal (Veldhuis et al, 2001, Cassoti et al, 2005, Peperzak & Brussaard 2011).

Figure 17. Bench top flow cytometer (Becton Dickinson Canto II), an instrument to enumerate live and dead organisms <50 µm.

Figure 18. (a) Epifluorescence microscopic picture of a live phytoplankton cell. The red signal is due to the presence of chlorophyll-*a*, and (b) a dead phytoplankton cell with a yellow-green fluorescence of the nucleus after staining with SYTOX Green.

5.2.3 Counting total heterotrophic bacteria

The classical method for counting bacteria in many applications is based on plating on selective media. Unfortunately, for studies in the aquatic environment this approach is by far insufficient for various reasons (Gasol & Del Giorgio 2000). Therefore, the total bacteria concentration in fixed samples is accurately determined by flow cytometry using the DNA-specific stain PicoGreen (cf. Gasol & Del Giorgio 2000, Veldhuis et al. 1997).

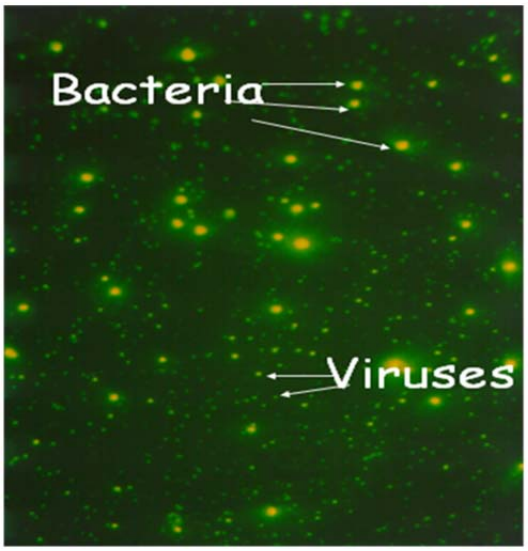

Figure 19. Epifluorescence microscopic picture showing bacteria and viruses in a natural water sample. The yellow-green fluorescence signal is DNA stained with a nucleic acid dye.

The dye PicoGreen is a green nucleic acid specific dye that only stains dsDNA, with little or no cross-over for ssDNA and RNA (Veldhuis et al, 1997). This makes the staining method ideal to for staining of DNA and therefore to determine bacterial abundance. Flow cytometric analysis shows a clear signal with an excellent signal to noise ratio and bacteria are made visible easily and distinguishable from viruses and larger organisms. This approach has extensively been compared with bacteria staining and counting using an epifluorescent microscope (Figure 19), resulting in nearly identical results. However, because the flow cytometer method is much faster (results are obtained within 100 seconds and over 100 samples can be analyzed per day), and highly reproducible this counting method is to be preferred above the far more time consuming and labour intensive microscopic observations.

5.2.4 Human pathogens

The samples for microbiological analysis are taken in special bottles of 300 or 600 mL and send to a special laboratory (Eurofins/ C-mark, Quality system: Testing RVA L154) for further analysis. All analyses are carried out according to NEN-ISO standards.

Escherichia coli

Analysis for *Escherichia coli* is carried out according to NEN-EN-ISO 9308-1 for the analysis of surface waters. For this the samples are filtered through membrane filters (pore size 0.45 µm) and these filters are incubated on a selective agar plate. Incubation is 4.5 ± 0.5 hours at 37 ± 1°C and then another 19.5 ± 0.5 hours at 44 ± 0.5 °C. After that the incubated filters are transferred on sterile filters soaked with Indol reagent. For colonies of *E. coli* this yields a red colour. These red colonies are counted and set into relation to the sample volume. Results are confirmed via a positive and a negative control. For the latter sterilized water is incubated like a regular sample and to confirm the results it may only yield less than 1 colony forming unit (cfu) per mL. The positive control uses a special strain of *E. coli* also incubated like a normal sample to confirm that this species can grow and form colonies on the used media.

Enterococci group

Analysis for this group is carried out according NEN-EN-ISO 7899-2. For this the samples are filtered through membrane filters (pore size 0.45 µm) and these filters are incubated on a selective agar plate. Incubation is 44 ± 4 hours at 36 ± 2°C on Slanetz & Bartley medium. After that red and pink colonies are counted. If the presence of enterococcus bacteria can be suspected after the colour of the colonies the filter is transferred to a pre-heated, selective agar plate and incubated for another 2 hours at 44 ± 0,5 °C. After that the medium is examined whether or not a brown to black colour can be found in it. Results are confirmed via a positive and a negative control. For the latter sterilized water is incubated like a regular sample and to confirm the results it may only yield less than 1 colony forming unit (cfu) per 100 mL. The positive control uses a strain of *Enterococcus faecium*.

5.2.5.2 PAM measurement for total phytoplankton viability

The photochemical efficiency of photosystem II is an indicator of the physiological 'health' condition of phytoplankton cells. It is a bulk variable that is measured using a Pulse Amplitude Modulated (PAM) fluorimeter (Schreiber et al 1993, Figure 20). The simple fluorescence ratio Fv/Fm gives a qualitative indication of the photosynthetic efficiency of the phytoplankton community. In addition, the maximum fluorescence value Fm is an indication of phytoplankton biomass.

Prior to the measurement the sample is kept in the dark for at least 30 minutes. 3 mL of unfiltered sample water (control and treated, each in triplicate) are filled into a glass cuvette and analysed within 2 minutes. In the case of a high photosynthetic efficiency of the bulk phytoplankton community, samples can be filtered using 50 and 10 µm Hydrobios™ gauze to determine the exact size class of the viable phytoplankton fraction.

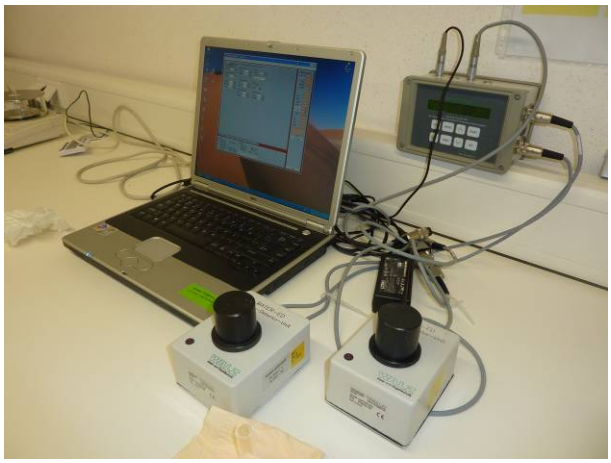

Figure 20. PAM fluorimetry, a fast method to determine (bulk) phytoplankton biomass and the physiological condition of the photosynthetic apparatus of the cells.

5.2.5.3 Counting phytoplankton <10 µm

Organisms in this size class will be analyzed via flow cytometry, as explained for the 10≤µm<50 phytoplankton size class.

6 Quality Assurance/ Quality Control (QA/QC)

The Quality Management Program (QMP) in section 1 of this project plan addresses the quality control management structure and policies of the test facility.

Sampling and analysis standard operating protocols (SOPs) contain QA/QC measures where applicable. The SOPs are listed in section 3 of this project plan.

6.1 Ballast water tests

For all ballast water test scenarios piping and instrumentation diagrams are available. Prior to each test a tool box meeting is held to ensure that the proper procedures are followed during intake and discharge. During the ballast water project all samples are taken, stored and analysed according to the dedicated SOPs. Prior to all tests sample codes are assigned following the rules explained in Table 4.

Because the test site is within very short distance of the main NIOZ building all samples containing fresh and live material are immediately transported to the laboratory for direct analysis. The sample storage flasks as well as cryovial boxes are labelled with the same coloured labels and codes. Samples that are fixed for long-term storage are stored in specifically designated refrigerators (4°C) and freezers (-20°C, -80°C).

The samples for microbiological analysis of the presence and number of human pathogens will be taken in special bottles of 300 and 600 mL and sent to "Eurofins/C.mark" in Heerenveen (accreditation certificate: RvA lab. no. L043). The samples will be transported immediately after sampling using a cooled transport container (4 °C).

6.2 Laboratory analyses

The analyses of abiotic and biological variables are described in general in other parts of this project plan. Detailed descriptions of each analysis are available. These Standard Operating Procedures (SOPs) of sampling, sample storage, sample analyses, data analyses and data management are part of the NIOZ Ballast Water QMP. Specific quality assurance and quality control measures are contained in each SOP.

6.3 Data analysis

The sample codes assigned a priori to the harbour tests (Table 4) are also used in data handling, i.e. the transfer of data from laboratory instruments to Excel™ files, dedicated to specific analyses. All data files are collected on a NIOZ network disk that is backed-up at least once a day. The separate data files are combined in one Excel™ file in which all appropriate calculations for D-2 and G8 will be conducted. The data on the NIOZ network disk are accessible to authorised NIOZ test facility personnel only.

Statistical analyses will be performed in Excel™ version 14. Additional analyses will be performed in either SYSTAT version 13 or Primer version 6. SYSTAT and Primer allow for more sophisticated statistical analyses of the BWTS' performance than the t-tests that are recommended in §2.3.37 of the G8-guidelines. The scientific hypothesis that will be tested, the so-called null-hypothesis, is that there are no differences between treated and control water samples.

Quality data are compiled in tables, and when possible visualised in diagrams such as, for instance, Shewhart control charts.

NIOZ will report the total number of tests that were needed to meet the D2-standard five times for each salinity range.

Table 5. Example of the assignment of coloured codes prior to tests. Sample flasks contain the appropriate code on a coloured label to prevent misidentification. T0 is the day of treatment and Tx is the x^{th} day of sampling. Usually x = 5 at discharge. Test numbers are Roman numerals (I, II, III, etc.) using a new number for every test (e.g. I to X). Replicates are denoted with normal numbers (1, 2, 3, etc.).
The example codes I-T5-M(1-4)-1 is, for each manufacturer, the first replicate sample of Test I on day 5 (T5).

Sample	Quay	Code	Label colour
Wadden Sea (harbour inlet)	Navicula	WSn	Red
Wadden Sea (harbour inlet)	Pelagia	WSp	Red+Orange
Control tank (T0)	Navicula	Cn	Green
Control tank (T0)	Pelagia	Cp	Green+Red
Treatment tanks for T0 to Tx:		e.g.:	
Manufacturer 1	Navicula	I-T5-M1-1	Orange
Manufacturer 2	Navicula	I-T5-M2-1	Yellow
Manufacturer 3	Pelagia	I-T5-M3-1	Purple
Manufacturer 4	Pelagia	I-T5-M4-1	Blue

7 References

Cassoti, R., S. Mazza, C. Brunet, V. Vantrepotte, A. Ianora & A. Miralto (2005) Growth inhibition and toxicity of the diatom aldehyde 2-trans, 4-trans-decadiental on Thalassirosira weissflogii (baciillariophycea). J. Phycol. 41: 7 - 20.

Falcioni, T., S. Papa, and J. A. Gasol. 2008. Evaluating the flow-cytometric nucleic acid double-staining protocol in realistic situations of planktonic bacterial death. Applied and Environmental Microbiology 74: 1767-1779.

Gasol JM, del Giorgio PAD (2000) Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Sci. Mar.* 64:197 - 224

Gregori, G. and others 2001. Resolution of Viable and Membrane-Compromised Bacteria in Freshwater and Marine Waters Based on Analytical Flow Cytometry and Nucleic Acid Double Staining. *Appl. Environ. Microbiol.* 67: 4662-4670.

Jeffrey SW, Mantoura RFC, Wright SW (1997) Phytoplankton pigments in oceanography. In: S.W. Jeffrey, R.F.C. Mantroura and S.W. Wright (eds) Phytoplankton pigments in oceanography: guidelines to modern methods SCOR-UNESCO, Paris pp. 661.

IMO (2005) Guidelines for approval of ballast water management systems (G8). Annex3 Resolution MEPC.125(53) Annex: Parts 1,2,3 and 4.

IMO (2008) Guidelines for approval of ballast water management systems (G8). Annex 4 Resolution MEPC.174.58.

Kraay G, Zapata M, Veldhuis MJW (1992) Separation of chlorophylls c1, c2 and c3 of marine phytoplankton by reversed-phase-C18-high-perfomance liquid chromatography. *J. Phycol.* 28:708 - 712

Mackey MD, Mackey DJ, Higgins HW, Wright SW (1996) CHEMTAX- a program for estimating class abundances form chemical markers: application to HPLC measurements of phytoplankton. *Mar. Ecol. Progr. Ser.* 144:265 – 283.

Peperzak, L, & CPD Brussaard. 2011. Flow cytometric applicability of fluorescent vitality probes on phytoplankton. *J. Phycol. 47*: 692-702.

Reinthaler T, Herndl GJ (2005) Seasonal dynamics of bacterial growth efficiencies in relation to phytoplankton in the southern North Sea. *Aquat. Microb. Ecol.* 39:7 - 16

Schreiber U, Neubauer C, Schliwa U (1993) PAM fluorimeter based on medium-frequency pulsed Xe-flash measuring light: A highly sensitive new tool in basic and applied photosynthesis. *Photosynth. Res.* 36:65 - 72

Shapiro HM (2003) Practical flow cytometry. John Wiley & Sons, Inc. New Jersey.

Veldhuis MJW, Cucci TL, Sieracki ME (1997) Cellular DNA content of marine phytoplankton using two new fluorochromes: taxonomic and ecological implications. *J. Phycol.* 33:527 - 541

Veldhuis MJW, Kraay GW (2000) Application of flow cytometry in marine phytoplankton research: current applications and future perspectives. *Sci. Mar.* 64:121 – 134.

Veldhuis MJW, Kraay GW, Timmermans KR (2001) Cell death in phytoplankton: correlation between changes in membrane permeability, photosynthetic activity, pigmentation and growth. *Eur. J. Phycol.* 36:167 – 177.

Veldhuis MJW, Fuhr F, Boon JP, Hallers-Tjabbes C.C. (2006) Treatment of ballast water; how to test a system with a modular concept? *Environ. Technol.* 27:909 – 921.

Section 3: Standard Operating Procedures (SOPs)

List of variables and related SOPs.
Supporting SOPs that are referred to in the listed SOPs are available on request.

Variable	unit	Reference (SOP)	Page
Salinity and Temperature	PSU (g/kg), °C	Salinity and Temperature 2012.2	46
pH	-	pH 2012.1	47
TSS and Particulate Organic Carbon	mg/L, mg/L	TSS-POC 2012.2	48
Dissolved Oxygen	% saturation	Dissolved Oxygen 2012.1	50
Dissolved Organic Carbon	mg/L	DOC 2012.1	53
Viable organisms ≥50 µm, including diversity	number per m^3, number of phyla and species	Mesozooplankton 2012.1	57
Phytoplankton (organisms 10-50 µm)	number per mL	Phytoplankton Canto FCM 2012.1 FCM Canto operation 2012.1 FCM Canto data processing 2012.3	59, 61, 64
Phytoplankton diversity	number of phyla and species	Koeman & Bijkerk b.v.; quality assessments in www.planktonforum.eu/	Koeman & Bijkerk, outsourced
Phytoplankton vitality (PAM fluorimetry)	Fv/Fm	Phytoplankton vitality PAM 2012.1	66
Phytoplankton vitality (SYTOX Green)	number per mL	Phytoplankton vitality SYTOX FCM 2012.1	68
Phytoplankton viability	+ or -	Plankton viability T5-incubation	71
Microzooplankton (organisms 10-50 µm) including diversity	number per mL and number of phyla and species	Microzooplankton 2012.2	69
Microzooplankton viability	+ or -	Plankton viability T5-incubation	71
Phytoplankton (organisms <10 µm)	number per mL	Phytoplankton Canto FCM 2012.1 FCM Canto operation 2012.1 FCM Canto data processing 2012.3	59, 61, 64
Heterotrophic bacteria	number per mL	Bacteria count PicoGreen 2012.1	73
E. coli	cfu per 100 mL	NEN-EN-ISO 9308-1	Eurofins C-mark, outsourced
Enterococci	cfu per 100 mL	NEN-EN-ISO 7899-2	Eurofins C-mark, outsourced

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STANDARD OPERATING PROCEDURE **DATE: 2012-09-05**
AUTHOR: Josje Snoek

Salinity and temperature 2012.2

Goal: Determination of salinity and temperature

MATERIAL & EQUIPMENT:

- Greisinger Digital Conductivity Meter GMH 3430 with Pt sensor
- Laboratory Salinity References: 3, 22, and 32 g KCl or NaCl/kg
- KCl or NaCl
- Dry oven, BINDER:ED 115
- Bucket, 12 litre with Ballast Water test water

Calibration:

- At intake and at discharge the GMH-3430 is calibrated for salinity against a lab-reference with the approximate ambient salinity value.
  For freshwater 3 g KCl (or NaCl) is dissolved in 997 g mQ
  For freshwater 22 g KCl (or NaCl) is dissolved in 978 g mQ
  For freshwater 32 g KCl (or NaCl) is dissolved in 968 g mQ
  KCl or NaCl is dried for overnight at 60 °C and cooled down in an exsiccator before weighing.
  Reference samples are made in a large volume and distributed over small ~ 60 ml plastic bottles.
  Each bottle is used once.
- Reading is correct if the difference with the reference solution is between +/- 5 %
- Correct the GMH-3430 reading by changing the Cell Correction factor.
  Press the Set/menu key for 2 sec and shortly press the same key a few times until Cell and a number between 0.40 and 1.00 appears on screen. Increase or decrease the correction factor value by pressing max (arrow upwards) - or min (arrow downwards)-key, set the value by pressing the Store/quit key.
  Check if the reading is correct, if not repeat the sequence.
  Record the measured values reference value before and after the correction in the harbour journal.
- Temperature is calibrated with a mercury precision thermometer, before and after each test cycle.
- Record the measured values mercury and sensor values in the harbour journal.

Measurement:

- Turn on the GMH-3430 by pressing the on/off key.
- Press Set/Menu key shortly until arrow in top of screen is at SAL.
- Salinity and temperature are measured directly in each sample bucket (3 per test) of BW-test water, together with pH measurement.
- Stir the salinity/temperature probe slowly through the sample record when the reading is stable.
- Record the values in the harbour journal.

LITERATURE:

- Operational Manual Conductivity Measuring Instrument version 2.0, Greisinger V.O.F.
- In 2004 at the time of the Ballast Water Management Convention **salinity** had no unit. Therefore, PSU as used in G8 is not a scientifically acceptable unit (http://www.oceanographers.net/forums/showthread.php?902-Salinity-Does-Not-Have-Physical-Units). Since 2010 salinity is in SI units: g/kg (http://www.teos-10.org/).

Royal Netherlands Institute for Sea Research

STANDARD OPERATING PROCEDURE **DATE: 2012-09-04**
AUTHOR: Josje Snoek

pH 2012.1

Goal: Determination of pH during harbour testing of ballast water treatment systems in fresh-, brackish- and seawater

MATERIAL & EQUIPMENT:

- Metrohm 826 pH mobile and Metrohm combined pH glass electrode: Unitrode 6.0258.010 with Pt1000 (for temperature).
- Metrohm buffer solutions pH 7 and 9, 30 x 30 ml
- Bucket (12 litre) with Ballast Water test samples

Calibration:
The glass electrode is calibrated at intake and discharge with 2 Metrohm buffer solutions.

- Turn on the pH meter by pressing the red button.
- Press OK to enter <param>, go with cursor to cal.settings/no off buffer, press 2 for a 2-point calibration. Leave <param> by pressing quit button
- Press the Call button.
- Open a new bag with buffer solution for each calibration.
  Start with pH 7 and stir slowly until instrument decides the reading is stable and asks for the next buffer.
- Repeat with pH 9.
- Information will be shown if the calibration in succeeded, if not repeat the whole process.
- Record the Calibration data in the harbour journal: press OK when <param> is shown at the bottom of the screen (or use arrow left or right until <param> is shown) , go with arrow-down to cal.data,

Measurement:

- pH is measured in each bucket (3 per test) with Ballast Water test water.
- Move the glass pH-electrode slowly through the water until the reading is stable.
- Be careful not to break the glass electrode.
- Record the value in the harbour journal

LITERATURE:

- See Applikon-Metrohm for specifications and instructions (http://www.metrohm.nl/PDFdownloads/MetrohmInfo/M-Info_1_05_E.pdf).

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STANDARD OPERATING PROCEDURE **DATE: 2012-09-06**
AUTHOR: Eveline Garritsen, Josje Snoek

TSS-POC 2012.2

Goal: Determination of Total Suspended Solids and Particulate Organic Carbon .

MATERIAL & EQUIPMENT:

- Balance
- Dried and pre-weighed GFC filters (Whatman, Glass microfiber filters, 47 mm, cat. no. 1822-047), in numbered 5 cm plastic petri dishes
- Dry oven 60°C , BINDER ED 115
- Exsicattor
- 1 litre graduated cylinder
- Filtration unit: Filtration frame with vacuum pump and four vacuum bottles with a filter holder
- Filter Forceps SS, Millipore
- Freshwater (MilliQ or mQ)
- Beaker
- Reference dried (60°C) mud as used in BWTS testing, stored in an exsicattor

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SAMPLING:

- During sampling in the harbour samples are taken in 1 litre square bottles, in triplicate for each test.
- Insert a pre-weighed filter into the Filter unit and write down the number of the filter in the harbour-journal and add the code of the test onto the petri dish.
- To avoid precipitation of TSS, mix gently but thorough and pore about 1 litre in one go into a 1 litre graduated cylinder.
- Read the volume and write it down in the harbour-journal, estimate the last ml.
- Filter the whole volume and rinse out the graduated cylinder with mQ, (also rinsing salt from the filter).
- When filtering is finished, turn off the vacuum pump and release the vacuum before you take the filter from the holder by using a pair of tweezers and put it back in the numbered petri disk.
- Store temporary in the fridge in the harbour, at the end of the day take all filters into the lab and store in a freezer (-80°C; -20°C is allowed for a short period) or directly put them in the Dry oven at 60°C to dry overnight.

ANALYSIS:

For TSS analysis GF/C filters are dried at 60°C for at least 8 hours and weighed again. The concentration of TSS per litre can be calculated from the sample volume and the weight difference of the filter before and after sampling. TSS is expressed in mg/l.

- Open the petri dish and put the cover under the bottom of the dish with the filter, and
  place them in the Dry oven at 60°C to dry overnight (min 6 hrs and max. 12 hrs.)
- After 6-12 hrs remove the petri dishes from the oven and store in a desiccator to cool-down under vacuum as long as needed.
- Weigh the GFC filters, note the filter number, weight and sample info in the lab-journal.
- Calculate the difference from the weighing before and after (in mg/l).

To determine the POC concentration the same filter is combusted overnight at 500°C and allowed to cool in a dessicator and weighed again.

- Fold the filters twice and place each filter in a numbered porcelain combustion dish.
  Keep a record of the filter number, sample info and combustion dish number.
- Combust overnight at 500°C and cool down in a desiccator.
- Weigh the filters and add the values to the record.

CALCULATIONS:

- Calculate TSS: weight after drying at 60°C – weight pre-dried empty filter at 60°C/sample volume
  TSS is expressed in mg/l.
- Calculate POC: weight after drying at 60°C – weight after combustion at 500°C/sample volume
  POC is expressed as mg C/l.

CALIBRATION:

- Accurately weigh approximately 50 mg reference dried mud
- Suspended in 1 L (graduated cylinder) 0.2 µm filtered seawater
- Treat this laboratory standard as a sample
- Repeat
- Calculate as a sample, and record the data with those of the test samples

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STANDARD OPERATING PROCEDURE **DATE: 2012-03-21**
AUTHOR: Josje Snoek

Dissolved Oxygen 2012.1

Goal: Determination of Total Dissolved Oxygen in seawater

MATERIAL & EQUIPMENT:

- Volume calibrated bottles (~120 ml)
- Dispenser bottles (3) (500 ml or 1 liter).
- Dispensers (3) max 2 or 5 ml per dose.
- Chemicals: MnCl2, NaOH, KI, H2SO4, KIO3.
- Magnetic stirring bars (many small, some large)
- Magnetic stirrer.
- Balance (g, mg)
- Hitachi U-1100 Spectrophotometer.

Introduction:
The chemical determination of oxygen concentrations in seawater is based on the method first proposed by Winkler (1888). In the Winkler method Oxygen is fixated by adding manganese chloride and alkaline iodide causing Oxygen to precipitate. This precipitate is subsequently dissolved by adding acid, causing a yellow coloured Iodine solution. The yellow colour can be determined directly with a Spectrophotometer.

Preparation of the Chemicals:
Reagent A (2 liter): MnCl2.4H2O 600 g/l.

- Weight 2 x 600 g MnCl2 in a 500 ml plastic measure cup
- Dissolve bit by bit, while mixing constantly with a spoon, in 1000 ml MQ in a 2 liter plastic beaker.
- When dissolved fill-up to 2 liter with MQ.
- Filter the solution through a coarse filter paper.
- Fill a dispenser bottle with reagent A , set the volume at 1 ml.
- Store the left over at room temperature in a plastic container.

Reagent B (2 liter): NaOH 250 g/l + KI 350 g/l.

- Weight 500 g NaOH in a 500 ml plastic measure cup.
- Dissolve bit by bit, while mixing constantly with a spoon, in 1000 ml MQ in a 2 liter plastic beaker.
- Add 2 x 350 g KI bit by bit while mixing until dissolved.
- Fill-up to 2 liter with MQ.
- Fill a dispenser bottle with reagent B , set the volume at 2 ml.
- Store the left over at room temperature in a plastic container.

Reagent C (1.8 liter):H2SO4 20 N (= 10M).

- Fill a 2 liter Erlenmeyer with 800 ml MQ.
- Place it in a bucket with ice-water.
- Slowly add 1000 ml H2SO4 96 % (end-volume 1800 ml).
- Mix after each addition.
- Fill a dispenser bottle with reagent C, set the volume at 1 ml.
- Store the left over at room temperature in a 2.5 liter brown glass flask.

Sampling:

- Remove all air bubbles from the tubing of the dispensers.
- Each BW-tank has a sampling point for small volumes. Rinse the tubing of the sample point by opening the valve for some time.
- Sample in triplicate in 120 ml volume calibrated bottles.
- Place the sampling tube at the bottom of a 120 ml volume calibrated bottle.
- Fill the bottle smoothly without air bubbles and overflow for 2-3 volumes.

- While the water is running, remove air bubbles by tapping the side of the bottle with the back of the glass stopper.
- Remove the tube from the bottle while the water is still running.
- Add 1 ml reagent A to the sample bottle with a dispenser (place the dispenser tube in the sample).
- Add 2 ml reagent B to the sample bottle with a dispenser (place the dispenser tube in the sample).
- Close the bottle with its own stopper (same number).
- Shake the bottle vigorously to ensure adequate reaction contact for the formation of the precipitate.
- Store the closed bottle under water in a (transport)container filled with cold tap water.

Spectrophotometrical determination:

Preparation.

- Turn on the Hitachi U-1100 Spectrophotometer 30 min before measurement.
- Place the left side of the tubing attached to the flow through cuvette of the spectrophotometer in a beaker with MQ.
- Attach a syringe to the other end of the tubing and suck the MQ through until it fills the syringe.
  Place the tubing with syringe in a bucket on the floor and remove the syringe. Fluid will keep running through the cuvette into the bucket. Check regularly if it is still running.
- Reset the blank value of the transmission at 456 nm when the reading is stable.
- Take the bottles you want to measure out of the storage container, without stirring the precipitate.
- Dry the bottles on the outside and under the rim of the stopper with a paper towel.

Measurement.

- Open the bottle by turning the stopper until it moves and slowly lift it from the bottle. If the bottle will not open use an attachment which fits the hexagonal top of the stopper to increase your grip.
- When open add 1 ml reagent C with a dispenser (do not place the dispenser tube in the sample).
  Watch out for spilling reagent C, it is a strong acid.
- Drop a stirring bar in the bottle and mix using a magnetic stirrer until precipitate is fully dissolved.
  The color of the sample should be brownish orange in different intensities depending on the amount of oxygen trapped.
- Take the left side of the tubing attached to the flow through cuvette of the spectrophotometer quickly out of the MQ, dry the tubing on the outside and place it in the sample.
- Let it run through the cuvette until the transmission reading is stable.

Calculation.

- To be able calculate the Oxygen concentration you need the slope and the intercept of a calibration curve with a Potassium Iodate Standard solution.
  For standard use: slope=0,0021 , intercept=0,0312 or determine them from your own calibration curve.
- Use the following equation for calculation of the Oxygen concentration of your sample.
  O2 umol/l = ((E456 sample-intercept)/slope)*(bottle volume ml +1)/(bottle volume ml-3)-1,05
  O2 mg/l = O2 umol/l * 32/1000
- For making a calibration curve prepare a Potassium Iodate Standard solution .
- Dry KIO3 for 6 hours at 180 °C, cool down in an excicator.
- Weigh ~2.5 KIO3g and dissolve in 250 ml MQ.
- Calculate the oxygen equivalent of the KIO3 solution:
  O2 umol/ul = 1.5* (ug KIO3*1000*1.00016)/(ml MQ*214) ~2.5 g KIO3/250 ml =~ 0.07 umol O2/ul.
- Prepare a blank sample by filling a volume calibrated bottle with sea water. Close the bottle and mix. Open the bottle and add the reagents in reversed order (C,B,A), one by

one, close the bottle and mix vigorously after each addition (be careful not to spill it on your clothes because of the strong acid).

- Open the bottle and add 100 – 700 ul Potassium Iodate Standard Stock solution to the blank sample and determine the Extinction at 456 nm and also measure a blank without addition of KIO3. Calculate the equivalent O2 concentration per volume KIO3-stock added:
  O2 umol/l = 1000* ul stock added *O2 umol/ul [stock]/(bottlevolume + ul stock added)
- Make a calibration curve (E456 vs O2 umol/l) and calculate the slope and the intercept.
- Report the calibration data with those of the test samples

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STANDARD OPERATING PROCEDURE **DATE: 2012-09-06**
AUTHOR: Eveline Garritsen

DOC 2012.1

Goal: This procedure describes how to analyse Dissolved Organic Carbon samples.

MATERIAL & EQUIPMENT:

- TOC-V, Total Organic Carbon Analyzer - Shimadzu
- ASI-V autosampler - Shimadzu
- 25 ml glass vials with screwcap (without septum)
- Pipets 5000/1000/200 µl
- tips for pipets 5000/1000/200 µl
- Platinised Alumina 1%, Pt Granular 0,85 to 1,2 mm, Elemental Microanalysis, 50 gm, BN 177644
- Silica (Quarz) Chips Granulaar 0,85 to 1,7 mm, Elemental Microanalysis, 50 gm, BN 176263
- Silica (Quarz) wool fine, Elemental Microanalysis, 50 gm, 502-177, B1102

- Deep Seawater Reference (CRM, 44-46 µM C, amp. 30 ml, Hansell Laboratory, University of Miami, Batch 11, lot#12-11
- Stock solution: 1000 ppm C(2.125 g of reagent grade potassium hydrogen phthalate that was previously dried at 105-120°C for about 1 hour and cooled in a desiccators before transferred in a 1 L volumetric flask and dissolved in milliQ water; 1000 ppm C=100 mg C/L).
  This stock solution is necessary for making reference concentrations of 0, 25, 50, 100 and 200 µM C.
- Hydrochloric Acid, 37-38% 2,5 l (max. 5 ppb Hg), 'Baker Analyzed', J.T.Baker, UN1789
- O,1 M HCl solution
- Anhydrone® ,Magnesium perchlorate, desiccant For Drying, 'Baker Analyzed', L.T.Baker, Cas No. 10034-81-8, Lot B27416
- MilliQ water

Procedure DOC:

The TOC-V instrument measures the amount of total carbon (TC), total inorganic carbon (IC) and total organic carbon (TOC) in water. 'Oxidative combustion-infrared analyses' is a widely-used TOC measurement method. The procedures related to TOC-Control V software system.

ANALYSIS: (Dutch)

Ongeveer **wekelijks** (na 120 ml aan injecties; 100 µl per injectie, ca. 5 tot 7 inj. per monster) moet de glazen kolom met katalysator vervangen worden:
- onder aan de kolom het T-stuk losdraaien (a)
- bovenaan de twee metalen schroeven links van inj.poort losdraaien (b)
- rechts van inj.poort de schroefverbinding met slang (TC, dragergas, O) (c)
- glazen kolom met injectiepoort van bovenaf uit het apparaat halen en daarna de glazen buis ook van onderaf uit injectieblok trekken (Pas op: Indien apparaat aan staat is de kolom zo'n 680 ºC!)
- deze buis apart neerleggen voor recycling
- op een schone en lege glasbuis twee streepjes zetten met zwarte viltstift voor markering van hoeveelheden (a.h.v. maatstreepjes op een kast)
- tot aan eerste streepje vullen met Silica quartz chips
- daarna vullen tot volgend streepje met Platinised Alumina
- vervolgens injectiepoort schoonspoelen, boven wasbak, met milliQ (denk erom dat de O-ringetjes er niet uitvallen, steek een pincet in de ringetjes en de opening, om ze op z'n plaats te houden)
- daarna injectiepoort droog blazen met perslucht
- schuif glasbuis van onderaf weer in injectieblok (tot hij stevig vast zit)
- doe de glasbuis met inj.blok terug op z'n plaats in het apparaat en zorg dat het slangetje(d) in het andere slangetje past; schroef (a) weer vast en zorg dat de dop de glasbuis ondersteund (de glasbuis komt onder druk te staan)

- daarna het perspex-plaatje van de inj. poort schoonspuiten met milliQ (op apparaat en leg er een tissue onder) en vervolgens onder de wieltjes op het inj.blok (e) schuiven; daarna schroeven (b) vastdraaien; let erop dat de bovenkant van het plaatje 'in lijn' staat met het achterste plaatje.

Als de kolom nog niet vervangen hoeft te worden, moet wel **dagelijks** het geklonterde Platinised Alumina uit de glasbuis worden gehaald (na afkoelen) m.b.v. een ijzerenstaaf en vervolgens moet er een beetje Platinised Alumina aan worden toegevoegd; dan ook het inj.blok schoonspoelen en droogblazen; tevens bovenplaatje van inj.blok schoon spuiten met milliQ.

dagelijks moet wel de "brug"(f) met Anhydrone® worden ververst:
trek de koppelingen (g) los van de brug en trek vervolgens de dop er af (aan Anhydrone kant);
schud leeg, haal de pluk glaswol eruit en laat de Tin korrels zitten; vervolgens weer een nieuwe pluk Silica Wool (goed aandrukken, Anhydrone erop 'gieten', schudden en de dop er weer op doen en de brug weer terugplaatsen
Buis (h) bijvullen met milliQ indien niveau te laag is (zie min-max markering)
controleer meterstanden van carriërgassnelheid (i), moet 110-130 zijn, manometers in apparaat en bij de zuurstofcylinder (j: ± 200 kPa)
Indien het glazen buisje (l), voor opvang van condenswater vol zit, dan twee van de drie koppelingen losmaken, het water in een bekerglas gieten en vervolgens de slangen weer aankoppelen
Als de buis (m) met 'halogen scrubber' (soort koperwol) donker van kleur is geworden, dan deze vervangen (niet te snel, hij is vrij prijzig).
Zet de TOC-V aan (knop voorop deur rechtsonder)
Kolom wordt 680 ºC; Detector zo'n 0,6ºC.

Acclimatisation

Voor je kunt beginnen met de analyse van de monsters moet het apparaat eerst geacclimatiseerd worden: gedurende ca. twee uur injecties met milliQ. Zorg altijd voor vers milliQ (slangetje van de ASI-V door dop van milliQ-vaatje). Gebruik file: aclimatization.t32.
- Programma TOC-Control opstarten
- Sample Table Editor aanklikken
- File aclimatization selecteren
- Edit, Delete, All (data)
- (connect), - use settings …

Background Monitor:
Temp.:
Fumace: 562 ºC Dehumidifier: 0,6 ºC

- (start), keep running, standby
- vial: 0 (= uit Erlenmeyer). Bij alle regels steeds vial 0 invullen (+ 21 regels)
- OK, (External Accid Addion -> niet)
- Start

(100 µl per injectie)
- View, Sample Window, (rechtermuisclick) Limits,
min. + max. aan Y-as aanpassen, b.v. -1 min en 5 max bij milliQ
- View, Methods, Instellingen

Methode bekijken: NPOC (Non Percal ….)
No. of inj. (is aantal injecties per vial) = 5/6
No. of wash = 3
SDMax = 0,1000
Max. integration = 02000
Sparge Time = tijd van vooraf doorborrelen (zuur toegevoegd, anorg. C gaat eruit)

Datum is naam van bestand
Save as: file name: 6 oktober 08 ballastwater.t32
- Edit, Delete data, All

Bij invullen scheme
- Insert, Auto Generate …, methode "sample.met"

No. of samples: (nog) 26 (40 totaal)
- OK
- Standby

- nr. van vial invullen (schema: Sparging/Acid Addition)
(Let op: geen Edit Acid Addition!)
- OK

Na + ¾ uur wassen met milliQ kun je de monsters draaien.

Sample Analysis:

Vul een formulier in voor het monsterschema. Hierop ook vermelden:
Filename, datum, projectnummer, naam gebruiker, informatie over kolom (nieuw of niet) soort monsters, gebruik van ijklijn, type aparaat, gebruik van standaard en eventuele bijzonderheden.
Monsters kunnen overnacht worden geanalyseerd met de autosampler.

- In carousel niet meer dan 50 tot 60 vials zetten.
- Altijd beginnen met milliQ (sampler heeft dubbele naald: eerste flesje wordt doorborreld, terwijl het tweede wordt geanalyseerd)
- In verband met het behoorlijke 'verloop' van de metingen (relatief gezien: op dit lage detectieniveau) is het aan te bevelen om wel drie ijklijnen te meten per serie, aan het begin, halverwege en aan het eind. Aan de hand van meetresultaten kan dan achteraf worden gecorrigeerd.
- - Na b.v. iedere zeven vials een 'schoonmaakvial' met 0,1 m HCL zetten.
- Het monsterschema, voor 18 monsters, ziet er dan b.v. als volgt uit:

1. milliQ	21. 12e
2. 0	22. HCl
3. 25	23. 0
4. 50	24. 25
5. 100	25. 50
6. 200	26. 100
7. HCl	27. 200
8. 1e	28. HCl
9. 2e	29. 13e
10. 3e	30. 14e
11. 4e	31. 15e
12. 5e	32. 16e
13. 6e	33. 17e
14. HCl	34. 18e
15. CRM	35. CRM
16. 7e	36. 0
17. 8e	37. 25
18. 9e	38. 50
19. 10e	39. 100
20. 11e	40. 200

- Ampullen met monsters openbreken (gebruik een opgevouwen tissue ter voorkoming van snijwondjes) door de top er met je duim af te breken, zet je duim op de witte stip.
- De inhoud van de ampul moet worden overgedaan in een 25 ml vial (5x gespoeld met milliQ) vanwege de te nauwe opening van de ampul en de afwijking in centrering van de naalden van de autosampler.
- Gebruik b.v.een 5 ml pipet en gebruik ong. 1 ml monster om de vial mee te spoelen. Er moet nl. ongeveer 18 ml in de vial zitten. Spoel de buitenkant van de pipetpunt goed af met milliQ en spoel nog 2x in Erlenmeyer met milliQ, alvorens het volgende monster wordt overgedaan.
- Om twee CRM vials te vullen, gebruik je 2 ampullen van 30 ml en spoel beide vials 3x met ca. 0,5 ml CRM-vloeistof
- Carrousel pas vullen met de vials indien deze allemaal gevuld zijn en er spoedig met het meten van de monsters kan worden begonnen (er moet niet veel tijd zitten tussen het acclimatiseren en de eigenlijke metingen, dus niet het apparaat lang stil laten staan.

Bereiding ijklijn uit stockoplossing
Stock: 1000 ppm C in koelkast. 500 ml maatkolven goed gespoeld met milliQ

0 µM C	-	0 µl stock
25	-	150
50	-	300
100	-	600
200	-	1200

Bij gebruik van electronische pipet, de pipetpunt schuin tegen de wand van de erlenmeyer houden (bij het 'uitspuiten') totdat de het 2e piepje en de trilling voorbij zijn!
Maatkolven met milliQ aanvullen tot 500 ml (onderkant meniscus raakt de lijn)
En daarna 2 ml HCl toevoegen. Het volume wordt dus 502 ml. Het volume van je monster is ook 20 ml + 4 druppels HCl.
Let op: dit phosphorzuur is erg stroperig en moeilijk te pipetteren.

Sample analyses
- : abort, are you sure … ? -> OK,
- Haal de cap van de carrousel, pak de carrousel eraf en zet de vials er goed in(= in de uitsparingen) en in juiste volgorde
- File, haal 'oude' file op, b.v. 8 oktober 08 ballastwater.t32
- Edit, Delete, All (alle meetresultaten worden weggegooid)
- : connect, even wachten (carousel gaat draaien en plunjer gaat op en neer)
- : 'stoplicht' (=start)
  In scherm de vial nummers invullen (b.v. 1 t/m 40, bij iedere regel)
- OK, start.

Calibration

Per serie twee of drie keer een CRM-standaard meten (= Certificate Reference Material) b.v. Deep Seawater Reference.

REMARKS:
- See also TOC-V user's manual and for data processing TOC-V Administrator's manual.

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STANDARD OPERATING PROCEDURE
Author: Frank Fuhr, Dennis Mosk, Eva Immler **2012-02-27**

SOP Mesozooplankton 2012.1

Goal:
This protocol describes sampling and analyzing of mesozooplankton in a ballast water context for land based testing. It refers to mesozooplankton as defined by the IMO D2-Standards as a size class > 50 µm. After treatment the allowed number of organisms in this size class at discharge is <10 viable org/m³.

MATERIAL & EQUIPMENT:
Harbour:

- 2 buckets 10 liter
- Bottle 2 liter
- Sieve 50µm (diagonal mesh size)
- Squeeze bottle
- 0.2 µm filtered seawater
- Plankton-net 50µm (diagonal mesh size) + net-beker (Hydrobios)
- 1000 liter tank (IBC)

Laboratory:

- Pipet 1-5ml
- Pipet tip
- Beaker-glass 300ml
- Sieve 30µm (diagonal mesh size)
- Squeeze bottle (0.2 µm filtered seawater)
- Bogorov or Borgorov-Gollasch dish
- Microscope 20x magnification, for determination 30- 60x (ZEISS V8 and V12)

CHEMICALS:

- Neutral Red; preparation of the solution:125mg standard Neutral Red stock in 250 ml demi water, (Neutral Red is added to the sample in a ratio to yield an end-concentration of 1:50.000).

SAMPLING:
Sampling of mesozooplankton for land-based certification tests of a ballast water treatment system is done in triplicates:
Day 0: 3x 1m^3 after treatment
3x 20 liters control after the pump
Day 5: 3x 1m^3 after treatment
3x 20 liters control after the pump

Harbour (control):

- Buckets are flushed 3 times with sample water
- Fill both buckets with 10 liters sample water
- 20 Liters are carefully filtered over a 50µm sieve.
- Organisms are carefully flushed from the sieve with 0.2 µm filtered seawater into a 2 Liter bottle. (mesozooplankton is sensitive to damage by nets and sieves)

Harbour (treated samples):

- Plankton-nets are flushed with fresh water
- The plankton-net is placed in an empty 1000 liter IBC with a closed net-beaker
- Fill the IBC with 1000 liters of sample water
- When during sampling the net is clogging,…..
- When the IBC is full, take the net slowly out of the IBC
- Organisms are flushed from the net into the net-beaker by using a squeeze bottle filled with filtered seawater (0.2 µm), and are carefully flushed from the net-beaker

into a 2 Liter bottle. Until further transport, store the bottles away from direct sunlight at a temperature comparable to the sample water.

Laboratory:

- The bottles are transferred back to the lab as soon as practical
- Samples are flushed into beaker-glasses and the volume is adjusted to any convenient manifold of 50ml and at least 150 ml to assure that the sample is not too concentrated for the organisms
- 2ml Neutral Red is added per 50 ml sample
- Stain for a minimum of 2 hours without direct sunlight and at a comparable temperature as the sample temperature
- After staining the sample is filtered over a 30 µm mesh net and carefully flushed with 0,2µm filtered seawater into a Bogorov or Bogorov- Gollasch counting dish

Analysis:

- Samples are counted complete (no subsampling)
- Subsamples are counted by at least two analysts and the individual counts are recorded
- Total amount of living organisms is counted and recorded on the plankton form
- Live dead determination is resulting from staining, structure and movement of the organism
- At minimum the phyla and abundance of the organisms present in the Bogorov counting dish is determined and recorded on the plankton form
- For more information see the *"NIOZ mesozooplankton booklet"* in laboratory F00-09
- At the start of the BWTS test season subsamples of 3 or more (old) samples are counted by three analysts; the individual counts are recorded; the difference between analysts should become <10%

REMARKS:

- From 2012 as well the Bogorov-Gollasch dish is used (chamber ends closed)

LITERATURE:

- Otto Larink & Wilfried Westerheide; (2006) *Costal Plankton- Photo Guide for European Seas,* , Muenchen
- Roger Harris; (2000) '*ICES zooplankton methodology manual*';, San Diego
- Zetsche E-M, Meysman FJR (2012) Dead or alive? Viability assessment of micro- and mesoplankton. J Plankton Res 34:493-509.

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STANDARD OPERATING PROCEDURE **DATE: 2012-02-23**
AUTHOR: Eveline Garritsen

PHYTOPLANKTON Canto FCM 2012.1

Goal: Determine the abundance of phytoplankton cells in Ballast Water samples.

MATERIAL & EQUIPMENT:

- Nalgene bottles, 1 litre
- Flowcytometer: BD Canto II
- FCM tubes

SAMPLING:

- Three 1 litre bottles are filled during (de)ballasting (at the begin, middle and end of filling/deballasting the ballastwater tank).
- Store the bottles is the fridge till analyzing time.
- Take a representative sample of approximately 3 ml of every bottle in a FCM-tube.
- Put the tubes in the carousel (begin at position 1, and there should be no open places between tubes of different treatments).

ANALYSIS:

- Start up protocol Canto: see protocol "SOP Canto".
- User: Ballastwater -> password: *ballast*
- Open protocol: Phytoplankton (dub. click -> boekje open)
  Settings:

	Voltage	Threshold
FSC	25	
SSC	198	
FBG	534	
FBO	394	
FBR1	390	200
FBR2	547	
FGO1	400	
FGR2	453	
FVB	341	
FVG	482	

- Click on **syringe** (=new specimen)
- **Rename** (right click on specimen)
- **Experiment – Experiment layout**
  Tab Acquisition – Events to record: fill in 10,000,000
  - Stopping time: 300
- **Carousel – Carousel setup**
  - Fill in carousel number (1,2,3 or 4 under Carousel ID)
  - Recording Delay time: 3 sec.
  - Mix Settings: v start of carousel mix
    v interim mix after every 1 v tube
    mix duration 3 sec
  - v Tube Pressurization Error Handling (Current Run)
  - v Show error and wait
  - OK

- Acquisition dashboard: **Flow rate: medium**
- **Run Carousel**
- Every time you have to measure the flowrate with trucount beads:
  Put 1 ml milliQ in a tube with n counts in it (see label on package)
  Use protocol 'Trucountbeads" and measure at medium flowrate for 60 sec.
  (Fill in on the Excell file"FACS Canto TruCount log.xlsx"with values of flowrates)

- **Measure flowrate**

- Rinse the FCM according to the protocol "SOP FACSCanto"

CALCULATIONS:

- Put data on your computer by using an USB stick. Data processing should be done with the program FCS express.
- Flowrate: Total count (measured)= count gate p1 + 2 x count gate p2
  n = count per tube (see label on package)
  Flowrate (µl/min)= (Total count/n) x 1000

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STANDARD OPERATING PROCEDURE **DATE: 2012-02-24**
AUTHOR: Anna Noordeloos

FCM Canto operation 2012.1

Goal: To operate and calibrate the Canto flow cytometer

MATERIAL & EQUIPMENT:

- BD Canto II Flowcytometer

Starting up

Switch the machine on with the green button on the left side of the flowcytometer.
It takes the machine 5-7 minutes to warm up.

- Switch on the computer. The window '*Log On to Windows'* appears. *Username: Administrator*. The password is BDIS →OK
- Click twice on the icon BD FACSDiva Software.
- Wait until the text *'The system is ready'* appears in the middle of the screen in the Cytometer window.
- Cytometer → Fluidics Startup → OK
- During Fluidics Startup you need to get the air out of all the fluidic filters on the wet car. You do this by turning the white knob on top of the filter anticlockwise while the liquid is flowing through. Keep the white knob open till liquid is coming out.
- Window *'Startup Status'* appears *Fluidics Startup is complete. The system is ready'* → OK
- Open the instrument and check the flowcel for air bubbles. In case of air bubbles Cytometer → Cleaning Modes → De-gas flow cell
- Window *'De-gas Flow Cell is complete'* → OK

Cytometer Setup & Tracking beads (CST)

Beads: BD Cytometer Setup & Tracking Beads

- Mixture of 2 and 3 µm beads with different intensities
- Type Lot ID
- Check met beads dagelijks uitvoeren duurt ca. 10 minutes
- Cytometer → CST
- Page '*Cytometer Setup and Tracking'* appears.
- Right top screen: Characterize: Check Performance → Run → OK
- Fill a flow cytometer tube with 0.35 ml mQ water
- Mix the bead vial by gentle vortexing
- Add one drop of BD Cytometer Setup & Tracking Beads
- Vortex the solution
- Store the solution in the dark and make this solution daily fresh
- Vink on Load Tube Manually, you find this directly under Run in Setup Control
- Window 'Cytometer Setup and Tracking' appears → OK
- Unload the tube
- After the calibration the report appears
- 'Cytometer Setup and Tracking' → View report
- Please watch the variance bright beads CV<6%, when not ok the values are in red
- File print
- After you've run the CST beads click on Performance Tracking. You'll find an overview of the last CST beads files.
- Close window CST

- Check performance
- CST mismatch window appears
- Click on middle option *'Use CST Settings'*

Trucount beads

Get a tube of Trucount beads from the drawer left of the Canto flow cytometer

- Read the number of beads from the bag and write it on the tube
- Add 1 ml of filtered seawater to the tubes
- Click on the book Trucount beads
- Right mouse button → Duplicate without data
- Rename the experiment with the correct date
- Open specimen
- Run first the tube with Trucount beads on Low flow speed for 180s
- Continue with med flow speed for 60s
- Finish with high flow for 60s
- Please, write the number of beads you've analyzed in the log book on the computer
- In Browser click on already made folder → right mouse button → Copy
- Go to Administrator → right mouse button → Paste
- Change in Inspector the name of the copied experiment
- Threshold FL-5 660/20 (rode laser) value: 1200
- Change to FL-1 530/30 (blue laser)
- In left 'kantlijn' Browser you find arrows in front of each tube. If you want to remove a tube you need to click on the arrow left en delete.
- Carousel setup → p.124 manual
- Carousel ID 1

- If you change from global to normal worksheet you can combine several samples.
- Select in browser all samples.
- Draw a plot in normal worksheet.

- Experiment layout → Acquisition

- Enter Events to Record → click

- 60 s measurement
- recording delay time 3 sec
- 'aanvinken' start of carousel mix
- 'aanvinken' interim mix after every 1 tube, mix duration 3 sec
- Cytometer → Degas flowcel and bubble filter (waar zit het bubble filter?)

Replace sheath vessel:

When sheet vessel is replaced:

- Cytometer → Cleaning modes → Prime after Tank refill (voor ontluchten) → FACSFlow → OK

- Cytometer Setup and Tracking
- Setup Control Research Use Only
- Define Baseline (wordt per machine 1x gedaan of na veranderen filter configuratie)
- Load tube Manually
- Flowrates LOW: 12, MED: 60, HIGH:120 ul/min (sample pressure:4.7)
-
- CST Mismatch
- Kies altijd Use CST Settings Deze melding krijg je alleen na een baseline calibratie.

- Experimenten aanmaken in Browser
- Inspector → Folder
- Symbool boekje aanklikken
- Via Inspector naam geven, recht muisknop
- Name: Beads
- 'aanvinken' Use global cytometer setting
- Click in Browser 'Cytometer settings'
- Wich detectoren ga ik gebruiken

- Inspector
- Global Sheet
- Name
- Number of Pages
- Hoe meten: 'spuitje' New specimen
- Via Inspector naam aanpassen
- Aquisition Dashboard gebruik je voor starten / stoppen metingen
- Acquire Data
- Sample beschrijving dmv Inspector

Tube manually

Acquisition Dashboard

- Right mouse button
- Show all
- Basic Control
- Manual

Rinse needle

- Cytometer → Cleaning modes → SIT flush

Statistics

- Statistiek → Click on plot → Right mouse button → Create statistic view

Batch analysis

- Click on booklet (Experiment) → right mouse button → batch analysis → Manual dan kan je nog gates aanpassen
- Plotjes saven als pdf. Je kan ook plotjes slepen naar excel sheet.

Shutdown procedure

- Fill four tubes with the following solutions: FACSClean, milliQ, FACSRinse, milliQ
- Cytometer → Cleaning Modes → place tube under needle → Clean Flowcel
- Repeat this step for all the tubes.
- Cytometer → Fluidics Shutdown→ File → Quit → Shutdown

In general:

- BD Technische dienst 020 582 9424
- Tube empty, air in system, no problem
- Optimale temperature for the flow cytometer 16C-31C
- Doesn't function well <5C and >40C when exposed for longer times.
- Power laser: 20 mW
- Pressure in system: 4.5 psi in system
- Speed particles trough flowcell at 6 m/s
- Maximal flowspeed 10.000 events/sec, if higher than coincidence. "melding' abort frequentie

- info.benelux@europe.bd.com
- CST beads, Ref. 641319: 119 euro per kit (21.03.11)
- Trucount tubes (50 tests), Ref. 340334: 157 euro (21.03.11)
- BD FACS Shutdown Solution, Ref. 334224

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STANDARD OPERATING PROCEDURE **DATE: 2012-09-06**
AUTHOR: Josje Snoek, Louis Peperzak

FCM Canto data processing 2012.3

Goal: Calculation cells/ml from CANTO-data (based on new FCS Express lay out).

MATERIAL & EQUIPMENT:

- BD Canto II Flowcytometer
- FCS express 4 (FCM analyses program); the lay-out for this software has been adjusted after size and concentration measurements of beads and phytoplankton cultures.

Exporting data from the CANTO

- Switch on the computer. The window '*Log On to Windows*' appears. *Username: Administrator*. The password is BDIS →OK
- Double click on the icon BD FACSDiva Software on the Desktop. *Username: Ballastwater*, password: ballast→OK
- Open BW-2012-test-results and select Stephan Gollash folder.
- Go to File/Export/FCS files, a Export FCS Files- window will open, press OK.
- Put the BW-USB in the computer and browse to the USB stick, select a folder,

  press Choose Directory, make a file name and press Save.
  Exporting will start.
- When exporting is ready close the CANTO program, take out the USB and turn of the computer.
- Copy the files to Flowcyt/BW-2012/Gollash Go-consult/ FCM Files/ Facs Canto.

Processing data with FCS-express

- If you don't have FCS-express on your computer already, copy the FCS-express.exe shortcut from the bio(L:)/ FCS/ FCS Express.exe –Shortcut (version 4).
- Open FCS-express version 4 with the shortcut on your computer.
- A empty Layout will appear
- Left mouse click on the round symbol, with nr 4 in it, on the top left side of the FCS screen.
- Open Layout, browse to Flowcyt/BW-2012/Gollash Go-consult/ FCM Files/ Facs Canto/ select BW-2012-CANTO-Phyto-SG-Louis-16-8-2012.fey
- Import the data into the Layout: go to Batch/Data List
  Select all the files and press on the red cross to erase all data.
  Press on the green plus and browse to Flowcyt/BW-2012/Gollasch Go-consult/ FCM Files/ Facs Canto open the folder(s) with your data, and select the .fcs files, press open.
  Select the first file in the list, go to the header of the Data List window and press Change File and press Change Data On All Plots. Close the Data List window.
- Check if all data fits well into the gates, go to Data and Click on Next or previous to check all selected files.
- If all files are OK, go to Batch/Batch Actions, right-mouse-click Excel (Column Mode), go to Properties, go to Save to a new fi and change the name of the file or even change the directory by browsing with the yellow folder sigh right of the window with the file name. Press OK
- Do the same with Export To Powerpoint. Choose the same name as the Excel file, press OK.
- Go to Batch/Run and all data will be calculated and stored.
- Repeat the same with the files of the True Count Beads (TCB) to calculate the flow rate.

Use the FCS-Layout for TCB beads, go to Flowcyt/BW-2012/Gollasch Go-consult/ FCM Files/ Facs Canto select BW-2012-CANTO-TCB-GS.fey.

- Open the excel Phyto-output of FCS express and copy all data into a FCM-data-calculation-file.
- Open the excel TCB-output of FCS express and copy counts of single and double counts into a
  flow rate-calculation-file. Calculate flow rate and Phyto-counts/ml see below.
- Examples of data calculation files:

FCS-OUTPUT-TCB-Layout

Trucount- I-II-K-T5_High 1.fcs compensated	single	1510	double	158
Trucount- I-II-K-T5_High 2.fcs compensated	single	1482	double	162
Trucount- I-II-K-T5_Medium 1.fcs compensated	single	792	double	84
Trucount- I-II-K-T5_Medium 2.fcs compensated	single	814	double	81

EXCEL calculation sheath flowrate

TCB batch # beads	volume sw (ml)	counting time (s)	TCB counts single	dubble	total	flowrate ml/min	flow rate	average	sdtdev
50602	3	60	1510	158	1826	0,108	high	0,108	0,001
50602	3	60	1482	162	1806	0,107	high		
50602	3	60	792	84	960	0,057	med	0,057	0,001
50602	3	60	814	81	976	0,058	med		

Total = single+(2* double); Flowrate =(total*60/counting time)/(TCB batch # beads/volume sw)

Xxxxxxxx-Phyto-Cp-T5

				5			
sample date	sample info	flowrate	flowrate ml/min	counted ml	FCS-OUTPUT	FCS-OUTPUT Large #	count/ml Large
17-04-12	I-Cp-T5-1	high	0,108	0,54	I-Cp-T5-1	102	189
17-04-12	I-Cp-T5-2	high	0,108	0,54	I-Cp-T5-2	140	260
17-04-12	I-Cp-T5-3	high	0,108	0,54	I-Cp-T5-3	167	310

Counted ml = flowrate * min counted (Phyto=300sec=5 min); count/ml = FCS OUTPUT #/counted volume.

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STANDARD OPERATING PROCEDURE **DATE: 2012-04-11**
AUTHOR: Eveline Garritsen, Josje Snoek

Phytoplankton vitality PAM 2012.1

Goal: Measuring photosynthetic activity of phytoplankton

MATERIAL & EQUIPMENT:
- PAM-Control, Walz
- WATER-ED Emitter-Detector-Unit, Walz
- Water-K, Quartz glass cuvette
- stopwatch

CHEMICALS:
- 0.2 µm filtered seawater

SAMPLING:
- Place the sample in the dark for at least half a hour

ANALYSIS:
- Switch on the computer.
- Start WinControl (shortcut on the desktop in the centre).
- Select PAM settings: File/Load
  - Select folder - double click on C, double click on BW folder.
  - Select file - BW-2, press OK.

The following PAM-settings will be loaded:

Measuring Light	
Freq.	**5**

Actinic light	
Int	**6**
Width	**00:10**

Clock	
Time	**00:20**
Item1:	**SAT-Pulse**

System Parameters	
F. Off	**4**
PM-Gain	**10, 15,20**
Out-Gain	**2**
ETR-F	**0.84**

Saturation Pulse	
Int	**10**
Width	**0.8**

Set		
X		Beeper
Mark		**A**

LED Amplitudes	
Meas.	**8**
Actinic	**12**

Far Red Light	
FR-Int	**6**
FR-Width	**00:10**

Status:	
X	Meas. Light
X	Photo-Multipl.

Blanc
- Fill the PAM cuvette with filtered seawater.
- Place the cover over the PAM cuvette
- Wait **1 min** (for the Ft signal to become stable).
- Check whether the Ft signal is zero. If this is not the case press the button left from the Ft signal (top of the screen right).
- Write down the F.Off's (values) for the three gains (10, 15 and 20).
- If you change the gain, you have to change the F.Off in the corresponding value.

- **Sample**
- Fill the cuvette with sample
- Wait **1 min.** before measuring the Ft signal

Note: The measurement is not reliable when the Ft value is below the 100 r.e. and above 1000 r.e. In this case you should increase respectively decrease the PM-Gain (versterkingsfactor photomultiplier). Write the PM-Gain down (10, 15 or 20).

- Press the F0 button
- Check for a straight horizontal line on the small graph on the bottom of the screen right. If not fill the cuvette with fresh sample and start again.
- Press the Fm button as soon as you see the F0 value written on the computer screen.
- Check for a signal in the small graph on the bottom of the screen with a flat top. Write down p (peak), x (flat line),~ (uncertainty)

Write down the F0, Fm and Fv/Fm ratio

Closing down the PAM:
Rinse the PAM cuvette with milli Q water
Close the window WinControl Report

The following message appears:
WinControl
*You will loose report, chart and kinetics data by quitting WinControl unless they were saved in *.csv/*.txt, ".pcf and *.pkf files*
Really leave

Press OK

Switch off the laptop
Switch off the PAM Control

REMARKS:
The PHYTO-ED system is best suited for field and on-deck work. The system is particularly recommended for work with open ocean water at chlorophyll contents below 0.5 µg/l.
(from: Brochure Walz, 04.2004)
The Universal Control Unit of the PAM has serial number: UKEA0117.
The Water-ED Emitter Detector Unit (Red version) has: EDEE0196.

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STANDARD OPERATING PROCEDURE **DATE: 2012-09-06**
AUTHOR: Josje Snoek

Phytoplankton vitality SYTOX FCM 2012.1

Goal: Determination of the vitality of phytoplankton in Ballast Water samples.

MATERIAL & EQUIPMENT:

- Nalgene Square bottles with 1 liter BW-sample.
- Flowcytometer: BD Canto II
- FCM tubes for BD CANTO
- Sytox green

SAMPLING:

- After Phytoplankton-FCM measurements samples are taken from the same bottles. Mix carefully and pipet **1 ml** into a FCM tube, add **10µl Sytox green** to the wall of the tube.
  When 6 tubes are filled tap with the bottom of the tube on the table to mix the SYTOX droplet with the sample. Start a timer for 10 min after mixing the first sample.
  Incubate **for 10-15 min** in the dark at room temperature.
- Put the tubes in the carousel of the FACS-CANTO and start the analysis.
- Start a new series of 6 samples when the first 3 samples have been measured and repeat this until all samples are done.

Measurement:

- With SOPs: Phytoplankton Canto FCM and FCM CANTO operation
- Use Phyto-SYTOX protocol, measuring time is 200 sec.

CALCULATIONS:

- See SOP FCM Canto data processing
- Use FCS Express Layout for Phyto SYTOX.

LITERATURE:

Peperzak L, Brussaard CPD (2011) Flow cytometric applicability of fluorescent vitality probes on phytoplankton. J Phycol 47:692-702

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STANDARD OPERATING PROCEDURE **27-02-2012**

Isabel van der Star, Eva Immler

Microzooplankton 2012.2

Goal:
Microzooplankton comprises by definition organisms in the size range of 20-200µm. This protocol refers to microzooplankton as defined by the IMO D2-Standards as a size class 10 ≤ µm < 50 µm. Maximum allowed numbers of organisms in this size class at discharge are 10 viable org/ml. This size class includes phytoplankton and zooplankton species. Phytoplankton is quantitatively analyzed by flow cytometry. Flagellates and dinoflagellates include autotrophic, heterotrophic and mixotrophic species, while ciliates are heterotrophic or mixotrophic. Heterotrophic species miss chlorophyll fluorescence and cannot be analysed by flow cytometry.

MATERIAL & EQUIPMENT:
Sampling:

- 1 L bottle
- 4ml Lugol/ L

Laboratory

- 50ml bottles
- Low pressure vacuum pump (< 0.1 bar)
- Weighing scale
- Tube
- Pipettes (0.5- 5 ml; 100-1000µl)
- Pipette tips (0.5- 5 ml; 100-1000µl)
- Utermöhl cuvette of 5-6 ml (0,17mm bottom thickness)
- Inverted microscope

CHEMICALS:

- Lugol (100 KI, 50g I_2, and 100ml HAc in 1 liter demi water), see SOP Lugol plankton fixative
- Sodium thiosulphate 1% dissolved in demiwater
- Bengal rose

SAMPLING:
Harbour

- 1L bottles with 4 ml lugol are prepared (keep in the dark prior to filling)
- Per sampling point 3 samples of 1 liter each are taken (3 controls after pump and 3 after treatment) Bottles are filled without filtering (ciliates are highly sensitive to damage by filters)

Laboratory

- Samples need to settle down for at least 24 hours. They are placed in a dark solid closet to avoid light and vibrations
- After settling sample bottles are weighted without lid:
    - Full sample
    - Concentrated sample
    - Empty bottle
- Sample is concentrated by sucking the non-concentrated part of the sample by using a vacuum pump. A volume of max. 50 ml of the homogenized concentrated sample will be saved in a dark bottle.
- The concentrate is saved in a fridge for max. one year. At room temperature the sample can be stored for 6 months.

ANALYSIS:

- Samples need to be homogenized by mixing very carefully. Use a pipette with a big opening to assure that ciliates will not be damaged
- For the control sampling point T0: take 1ml out of all tree concentrated samples and mix carefully in a tube, take a subsample of 1ml and analyze in the Utermöhl cuvette.
- In general: analyze at least 1 ml per control sampling point and at least 3 ml per treated sampling point
- Place the volume in a tube and include one or more droplets of sodium thiosulphate to neutralize the Lugol. Then the sample is stained with a small droplet of Bengal Rose. This sample is placed in a Utermöhl cuvette. Fill this cuvette fully by filtrated (0.2 µm) and sterilized sea water.
- Analysis will be done after 2 hours of staining and settling down of the sampling
- Use a 0.17 mm cover glass over the cuvette to assure no movement in the cuvette itself
- Analysis of abundance and species determination to the lowest possible taxonomic unit is done by using an inverted microscope (200x objective, 400 and 600 for determination)
- For more detailed information see micro zooplankton booklet in the microscope room F00-05
- At the start of the BWTS test season subsamples of 3 or more (old) samples are counted by three analysts; the individual counts are recorded; the difference between analysts should become <10%
- A number of samples will be compared with counts made in the same sample by Koeman & Bijkerk

CALCULATIONS:

- Sample weight = full sample weight – empty bottle weight
- Concentrated sample weight= concentrated sample weight – empty bottle weight
- Ciliate count/ (sample weight/concentrated sample weight)* ml analyzed

REMARKS:

- Prior to 2012 4 full series of the certification tests done for a company in one year were analyzed. From 2012 onwards for the intake samples a mixture subsample of triplicates at every sampling point are analyzed for all certification tests. Same applies for the control samples of the discharge day. Treated discharge samples are all analyzed.

- Before 2012 the micro zooplankton data was entered and processed in the counting program 'Koeman en Bijkerk' (ecologic research and advising). From 2012 onwards data will be processed in Excel

- See separate SOP Plankton viability T5-Incubation

LITERATURE:

- Koeman, R.P.T., Esslink, K.Fockens, A.L. de Haan & G.L. Verweij. 2002. *Biomonitoring van microzooplankton in de Nederlandse zoute wateren 2000.* Rapport 2001-22. Bureau Koeman en Bijkerk, Haren
- Koeman en Bijkerk intern rapport genoteerde soortenlijst 2000-2010

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STANDARD OPERATING PROCEDURE **DATE: 2012-03-05**
AUTHOR: Louis Peperzak

Plankton viability T5-Incubation 2012.1

Goal: To measure the development of phyto- and microzooplankton in treated ballast water samples that were discharged after a five day holding period as a measure of viability. In UV-systems this means after a second UV-treatment on discharge. Samples for PAM and microzooplankton are taken on the first day of incubation and up to seven days, on working days only. On day 7 complete sampling is performed, including samples for phytoplankton, bacteria and microzooplankton.

MATERIAL & EQUIPMENT:

- 10 L polycarbonate bottle (Nalgene)
- Magnetic stirrer and stir bar (rotation 130 rpm)
- Climate room at ambient temperature and irradiance at 100 µmol photons m^{-2} s^{-1}; L:D = 18:6 (h:h)
- PAM fluorimeter
- Canto flow cytometer
- Inverted microscope
- 100 mL sample bottles
- Greiner tube 50 mL with black tape
- 2.5 mL cryovials
- Flow cytometer tubes
- 1 mL pipets

CHEMICALS:

- Phytoplankton nutrient stocks: N, P, Si (Eveline); add one bottle of each nutrient to 10L sample
- Lugol's iodine solution
- Formaldehyde-hexamine 18% (v/v)

METHOD:

- Incubate 10 L sample on T5 (discharge day) in the climate room; add nutrients and stir
- Sample on each work day for 7 days (T12)

SAMPLING:

- Pour 100 mL sample into a 100 mL sample bottle with 0.4 mL Lugol and store at 4°C in a refrigerator (microzooplankton)
- Pour 50 mL sample into a 50 mL Greiner tube
- Pipette 2x 1.5 mL(bacteria) en 3.5ml (phytoplankton) sample from the Greiner tube in two cryovials with 18% formaldehyde (100 µl – phytoplankton en 150 µl-bacteria),
- snap freeze
- store at -80°C (bacteria + phytoplankton)
- Store the Greiner tube 30 minutes at room temperature (PAM)

ANALYSIS:

- Measure phytoplankton viability with the PAM (separate SOP)
- Measure bacteria and phytoplankton after thawing of samples with the flow cytometer (separate SOP)
- Measure the microzooplankton concentration with an inverted microscope (separate SOP)

REMARKS:

- see separate SOPs for PAM and the measurement of bacteria, phytoplankton and microzooplankton
- not all samples will be analysed; this will depend on the microzooplankton concentration on discharge

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STANDARD OPERATING PROCEDURE **DATE: 2012-09-06**
AUTHOR: Josje Snoek

Bacteria PicoGreen count FCM 2012.1

Goal: Determination the concentration of heterotrophic bacteria in ballast water samples.

MATERIAL & EQUIPMENT:

- Nalgene Square bottles with 1 litre ballast water sample.
- Flowcytometer: BD Canto II
- FCM tubes for BD-CANTO
- PicoGreen, 100 µL commercial stock in 400 µL TE buffer

SAMPLING:

- Samples are fixed with formaldehyde and stored at -80°C
- After thawing add 1 mL sample to 10 µl diluted PicoGreen
  Incubate for 10- 30 minutes in the dark at room temperature.
- Put the tubes in the carousel of the FACS-CANTO and start the analysis.
- Start a new series of 9 samples when the first 3 samples have been measured and repeat this until all samples have been analysed.

Measurement:

- With FCM BD CANTO, see SOP FACS CANTO-Operation 2012.1 and FACS CANTO data processing 2012.3
- Use the BD Canto "Bact-SYTOX" protocol, measuring time is 100 sec.

CALCULATIONS:

- See SOP FCM-Data Processing with FCS Express 4.0 2012.1
- Use FCS Express Layout for Phyto SYTOX.